HIF dependent and independent transcriptional regulation of the human *PHD2* promoter

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Abbreviations

2-OG	2-oxoglutarate
aa	amino acids
AD	activation domain
AhR	aryl hydrocarbon receptor
AMP	adenosine monophosphate
AP-1	activating protein-1
ARD1	arrest-defective-1
ARNT	aryl hydrocarbon receptor nuclear translocator
ARNTL	aryl hydrocarbon receptor nuclear translocator like
ATF4	activating transcription factor-4
Bcl-2	B-cell CLL/lymphoma 2
bHLH	basic helix-loop-helix
BNC	basonuclin 1
CAIX	carbonic anhydrase 9
cAMP	cyclic AMP
Cap43	calcium activated protein 43
CBP	CREB-binding protein
CDF-1	cell growth defect factor 1
Cdr2	cerebellar degeneration-related protein 2
CH-domain	cysteine/histidine rich domain
ChIP	chromatin immunoprecipitation
CHX	cycloheximide
CITED2	CBP/p300-interacting transactivators with glutamic acid (E) and aspartic acid (D)-rich tail
COP1	constitutive photomorphogenesis protein 1 homolog
CREB	cAMP response element-binding protein
CTAD	carboxy-terminal transactivation domain
DFX	desferrioxamine
DIMP1	cyclin D binding myb-like transcription factor 1
DMOG	dimethyloxalylglycine

DSS	dextran-sodium sulphate
E	embryonic day
E1AF	E1A enhancer binding protein
EGL-9	egg-laying abnormal-9
EHF	ETS homologous factor
ELF	E74-like factor
ELG	ETS like gene
EMSA	electromobility shift assay
EPAS-1	endothelial PAS protein 1
EPO	erythropoietin
ER	endoplasmatic reticulum
ER81	ETS related protein 81
ERF	ETS repressor factor
ERG	v-ets avian erythroblastosis virus E26 oncogene related gene
ERK	extracellular signal-regulated protein kinase
ERM	ETS related molecule
ESE	epithelial specific ETS
ETS	E-twenty six (E26)
ETS	v-ets erythroblastosis virus E26 oncogene homolog;
ETV	ETS translocation variant (or ETS variant gene)
EWS	Ewing sarcoma RNA-binding protein
FEV	fifth Ewing variant
FIH	factor inhibiting HIF
FKBP38	FK506-binding protein 38
FLI1	friend leukemia virus integration 1
FRET	fluorescence resonance energy transfer
GABP	GA repeat binding protein
Gulo	L-gulono-1,4-lactone-oxidase
GLUT-1	glucose transporter 1
HBS	HIF-binding site
HIF	hypoxia-inducible factor
HLH	helix-loop-helix
HPH	HIF prolyl hydroxylase

HRE	hypoxia response element
HSP70/90	heat shock protein 70/90
ICAM-1	intercellular adhesion molecule 1
IGF1/2	insulin-like growth factor 1/2
ING4	inhibitor of growth family member 4
IPAS	inhibitory PAS protein
IRES	Internal ribosome entry site
IκB	inhibitor of NFκB
K_m	Michaelis Menten constant
LDHA	lactate dehydrogenase A
LIN	abnormal cell lineage
LOXL2	lysyloxidase like 2
MAPK	mitogen-activated protein kinase
MEF	mouse embryonic fibroblast
MEF	myeloid ELF1-like factor
MMP	matrix metalloproteinase
MW	molecular weight
N	asparagine
NDRG1	n-myc-down regulated 1
NERF	new ETS-related factor
NFκB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NLS	nuclear localization signal
NO	nitric oxide
N-OG	N-oxalylglycine
NOS	nitric oxide synthase
NTAD	amino-terminus transactivation domain
ODDD	oxygen-dependent degradation domain
OS9	amplified in osteosarcoma 9
p300	300 kDa protein, histone acetyl transferase
PAS	PER-ARNT-SIM
PDEF	prostate derived ETS transcription factor
PEA3	polyomavirus enhancer activator-3
PER	period circadian protein *(Drosophila)*

PGK	phosphoglycerate kinase
PHD	prolyl-4-hydroxylase domain containing protein
PI3K	phosphatidylinositol-3-kinase
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PNT	pointed domain
pO_2	oxygen partial pressure
PSE	prostate epithelium-specific ETS
PTEN	phosphatase and tensin homolog deleted on chromosome ten
pVHL	von Hippel-Lindau tumor suppressor protein
RD	repressor domain
REDD1	regulated in development and DNA damage response 1 protein
Ref-1	redox factor-1
RNAi	RNA interference
ROS	reactive oxygen species
SAP	serum response factor accessory protein
SENP	sentrin/SUMO-specific protease
SHH	sonic hedgehog protein
SIAH	seven in absentia drosophila homolog
SIM	single-minded protein *(Drosophila)*
SP1	specificity protein 1
SPDEF	SAM pointed domain containing ETS transcription factor
SPI	spleen focus forming virus proviral integration oncogene
SRC-1	steroid receptor coactivator 1
SUMO	small ubiquitin-related modifier
TAD	transactivation domain
TCA	tricarboxylic acid cycle (Krebs cycle)
TCF	ternary complex factor
TEL	translocation, Ets, leukemia
TGFβ	transforming growth factor β
TIF2	transcription intermidiary factor 2
TNF	tumor necrosis factor

UTR	untranslated region
VEGF	vascular endothelial growth factor
wt	wild-type
WT1	Wilms tumor 1

Summary

A disproportion between oxygen delivery and consumption leads to a restricted oxygenation of tissues. In a variety of pathologies like ischemia, stroke, inflammation and cancer this shortage of oxygen is a key feature. It enrolls a unique response that is based on the transcriptional regulation of hundreds of downstream target genes that promote eventually the adaption of cell metabolism. The maintenance of the oxygen homeostasis is centrally governed by *hypoxia-inducible* transcription factors (HIFs). Direct target genes of these transcription factors are increasingly expressed by binding of the HIF-complex to the *cis*-acting highly conserved consensus sequence 5'-RCGTG-3'; also referred to as hypoxia response elements (HRE). Heterodimeric HIFs consist of a tightly O_2-regulated α-subunit (in humans HIF-1α, HIF-2α or HIF-3α) and a constitutively expressed β-subunit (HIF-1β). In oxygenated conditions HIF α-subunits are continuously marked for proteasomal degradation through hydroxylation of two key prolyl-residues by prolyl-4-hydroxylase domain (PHD) oxygen sensor proteins. In hypoxic conditions the HIF-α subunit is stabilized and translocates to the nucleus where it forms the heterodimer HIF. Biochemically, the stabilization of the HIF-α subunit is explained by a reduced hydroxylation that is required for the interaction with the ubiquitin-ligase von-Hippel-Lindau protein (pVHL).

Two of three PHDs are transcriptionally regulated by HIFs. As a consequence HIF causes increased expression of PHD2/3 that compensates for their decreased enzymatic activity in hypoxia. In this negative HIF-PHD2/3 feedback loop we decided to focus on the oxygen sensor PHD2. PHD2 is widely considered as the main cellular oxygen sensor since, amongst other evidences, only the knockout of the *PHD2* (*EGLN1*) gene shows prenatal lethality in mice.

We regard the transcriptional regulation of the *PHD2* gene as important since the abundance of the PHD2 enzyme determines the above mentioned negative feedback loop. Therefore, we aimed to profoundly understand the transcriptional regulation by studying the *PHD2* promoter architecture and to elucidate further regulatory mechanisms of its activity.

We carried out consecutive truncations of the *PHD2* promoter and defined the minimal promoter region. By chromosome immunoprecipitation we could confirm on

an endogenous level that hypoxic PHD2 expression is predominantly mediated through HIF-1α rather than HIF-2α. Additionally we identified and cloned a 95 and 55 nucleotide *PHD2* promoter region encompassing a single HBS as highly conserved in several organisms and demonstrated high hypoxia-inducibility. To date, HIF is the only known transcription factor influencing *PHD2* gene transcription. However, various putative transcription factor binding sites were predicted in this conserved *PHD2* promoter region. By a mutation approach we could exclude the ubiquitous transcription factor Sp1 to be involved in basal or hypoxia-induced regulation of the *PHD2* gene although numerous predicted Sp1-consensus motifs suggested so. When motifs located 5' or 3' to the HBS were mutated, total abrogation of the hypoxic response was observed, but binding of the HIF-1 complex remained unaffected. This suggests that other transcription factors might contribute to hypoxic activation of the *PHD2* promoter.

In order to find out which other (co-) transcription factors might influence the *PHD2* promoter activity we established a synthetic transactivation screening where 704 arrayed transcription factors were analyzed for their influence on the PHD2 HBS (Wollenick *et al.*, *Nucleic Acid Res.*, *in press*). We found several family members of the activator protein-1 (AP-1) transcription factors, such as JUN and FOSB, and three ETS-transcription factors to be involved in the activation of the *PHD2* promoter. Most strikingly, the ETS-transcription factor ETS variant 4 (ETV4) showed, when overexpressed, not only impact on hypoxic *PHD2* expression but also on other well-known hypoxic target genes such as *PHD3* (*EGLN3*) and *carbonic anhydrase 9* (*CA9*). We hypothesize that ETV4 potentially increases the hypoxic activation of those promoters or elements that contain a distinct sequence architecture surrounding the HBS. HBSs that are similar to the PHD2 HBS seem to be preferentially super-induced by ETV4.

By mammalian two-hybrid and fluorescence resonance energy transfer (FRET) analysis we found evidence for formation of a complex between ETV4 and HIF-1/2α. Chromatin immunoprecipitation confirmed the recruitment of HIF-1α and ETV4 to the *PHD2* locus. Additionally, we could provide evidence that the co-activation of hypoxic target genes by ETV4 also has relevance for clinical data. *In vivo* data underlined that ETV4 expression strongly correlates with PHD2, HIF-1/2α and other hypoxic marker genes in 282 human tissues of breast cancer patients.

Although FRET data suggest a direct interaction, we hypothesize a trimeric complex composed of HIF:p300/CBP:ETV4. We carried out a thorough HIF-1α domain mapping and found that during the hypoxically induced HIF-1α:ETV4 interaction mainly the C-terminal activation domain is involved. Additionally, overexpression of CBP/p300-interacting transactivator 2 (CITED2), a competitor of HIF for the p300/CBP interaction, disrupted the ETV4:HIF complex pointing towards the involvement of p300/CBP. Factor inhibiting HIF (FIH) depletion provoked unregulated binding of HIF to p300/CBP and as a result the loss of oxygen-dependent suppression of the interaction between HIF and ETV4. Taken together, these experiments provided evidence for the cooperation between HIF-1α and p300/CBP in ETV4 binding.

Recent data provide indications that ETV4 protein is more abundant in hypoxic and in PHD2 knockdown cells while ETV4 mRNA levels remain unaffected. ETV4 protein levels were also increased when cells were treated with a PHD inhibitor. That might hint to a hydroxylation-dependent regulation of ETV4 through PHDs that is inhibited when the O_2-concentration is low or when PHDs are silenced.

In conclusion, this work demonstrated that a synthetic transactivation screening can unravel so far unrecognized transcriptional pathway interactions that also have implications on clinical data of different cancer specimen.

Zusammenfassung

Ein Ungleichgewicht in der Sauerstoffzufuhr und dem Sauerstoffverbrauch führt zu einer limitierten Oxygenierung des Gewebes. Eine Reihe von Erkrankungen wie z.B. Ischämien, Hirnschläge, Entzündungen oder Krebserkrankungen haben Sauerstoffmangel als Schlüsseleigenschaft. Bei der Sauerstoffunterversorgung wird zellulär eine evolutionär-konservierte Antwort ausgelöst, die die transkriptionelle Regulierung von mehreren 100 Genen zur Folge hat. Letztlich wird somit der Zellstoffwechsel an die veränderten Bedingungen angepasst und sichert das Überleben des Gewebes. Die Sauerstoffhomöostase wird hauptsächlich durch die hypoxieinduzierten Faktoren (HIFs) gesteuert. Direkte Zielgene dieser Transkriptionsfaktoren werden verstärkt exprimiert, indem der HIF-Komplex an *cis*-Elemente mit der hochkonservierten Konsensussequenz 5'-RCGTG-3' - auch Hypoxie-Response-Element (HRE) genannt - bindet. Die heterodimeren HIFs bestehen aus einer stark sauerstoffregulierten α-Untereinheit (im Menschen: HIF-1α, HIF-2α, HIF-3α) und einer kontinuierlich exprimierten β-Untereinheit (HIF-1β). Unter sauerstoffreichen Bedingungen werden die HIF-α-Untereinheiten stetig für den proteasomalen Abbau markiert. Dies geschieht durch die Hydroxylierung zweier Proline durch HIF-Prolyl-Hydroxylasen (PHDs), die als Sauerstoffsensoren fungieren. Unter hypoxischen Bedingungen dagegen stabilisiert sich die HIF-α Untereinheit und transloziert in den Zellkern, wo schliesslich das Heterodimer HIF gebildet wird. Biochemisch ist die Stabilisierung der HIF-α Untereinheit mit einer reduzierten Hydroxylierung zu erklären. Die Interaktion mit der Ubiquitin-Ligase von-Hippel-Lindau Protein (pVHL) benötigt jedoch die vorgängige Hydroxylierung der α-Einheit. Interessanterweise sind zwei der drei PHDs selbst durch HIFs transkriptioniell reguliert. Daraus resultiert eine durch HIF ausgelöste vermehrte PHD2/3 Expression, die die erniedrigte Enzymaktivität in der Hypoxie kompensiert.

In der beschriebenen HIF-PHD2/3-Rückkopplungsschleife haben wir uns auf den Sauerstoffsensor PHD2 fokussiert, da PHD2 als wichtigster zellulärer Sauerstoffsensor betrachtet wird. Das Primat von PHD2 als Hauptsauerstoffsensor wurde unter anderem durch einen Knockout in Mäusen bewiesen, der pränatal zum Tod der Embryonen führt.

Wir erachten die transkriptionelle Kontrolle des *PHD2* Genes als wichtig, da die Menge an PHD2-Enzym im entscheidenden Maße die zuvor genannte negative Rückkopplungsschleife beeinflusst. Eine tiefgehende Strukturanalyse des *PHD2* Promoters sollte dazu beitragen, seine Regulationsmechanismen besser zu verstehen.

Dazu führten wir eine Abfolge von Deletionen am *PHD2* Promoter durch und definierten so den Minimalpromoter. Mit Hilfe der Chromatin-Immunopräzipitation konnten wir auf endogenem Level zeigen, dass hauptsächlich HIF-1 die hypoxische Expression des *PHD2* Gens dominiert.

Darüber hinaus identifizierten und klonierten wir ein 95 und 55-Nukleotid langes *PHD2* Promoterstück, welches die alleinige HIF-Bindungsstelle (HBS) umspannt und in mehreren Organismen stark konserviert ist. Diese Promoterregion zeigte sich hochgradig hypoxieinduziert.

HIF ist bis dato der einzig bekannte Transkriptionsfaktor, der das *PHD2* Gen in seiner Transkription beeinflusst. Dennoch wurden in diesem konservierten *PHD2* Promoterelement mehrere mögliche Bindungsstellen für andere Transkriptionsfaktoren vorhergesagt. Durch einen Mutationsansatz konnten wir den ubiquitären Transkriptionsfaktor SP1 von der basalen oder hypoxieinduzierten Regulation ausschliessen, obwohl mehrere *in silico* vorhergesagte SP1-Konsensusmotive eine Regulation nahegelegt haben. Mit Hilfe von Mutationen der HBS flankierenden 5'- und 3'-Regionen wurde der Hypoxie-verursachte Anstieg der *PHD2* Promoteraktivität komplett unterdrückt, wenngleich die Bindung des HIF-1 Komplex an die HBS nicht betroffen war. Dies ist ein erneuter Hinweis auf die Existenz weiterer Transkriptionsfaktoren, die in der hypoxischen Aktivierung des *PHD2* Promoters involviert sein könnten.

Aus diesem Grunde entwarfen wir einen synthetischen Transaktivierungsansatz, in dem 704 Transkriptionsfaktoren auf ihren Einfluss auf die PHD2 HBS untersucht wurden (Wollenick *et al.*, *Nucleic Acid Res.*, *im Druck*). Bei der Analyse fanden wir einige Aktivatorprotein-1 (AP-1) Familienmitglieder, sowie drei ETS-Transkriptionsfaktoren, die in der Aktivierung des *PHD2* Promoters eine Rolle spielen. Ein besonders starker Effekt wurde mit der Überexpression des ETS-Transkriptionsfaktors ETS Variante 4 (ETV4) erreicht. ETV4 erhöhte nicht nur die hypoxische PHD2 Expression, sondern auch andere bekannte hypoxieinduzierte Zielgene. So zeigten sich z.B. *PHD3* (*EGLN3*) und die *Carboanhydrase 9* (*CA9*) als

stark ETV4-abhängig. Wir vermuten, dass ETV4 die hypoxische Aktivität solcher Promotoren oder Elemente steigert, die eine distinkte Sequenzarchitektur in der HBS-Umgebung aufweisen. Anscheinend werden jene HBSen, die der PHD2 HBS ähneln, bevorzugt von ETV4 superinduziert.

Ein mechanistischer Beleg der Komplexbildung von ETV4 und HIF-1/2α fanden wir in Mammalian-Zwei-Hybrid-Systemen und Fluoreszenz-Resonanzenergietransfer (FRET)-Experimenten. Chromatin-Immunopräzipitation bestätigte zusätzlich die Rekrutierung von HIF-1α und ETV4 an den *PHD2* Locus. Ferner konnten wir beweisen, dass die Ko-Aktivierung von hypoxischen Zielgenen auch medizinische Bedeutung hat. *In vivo* Daten bestätigten eine stark positive Korrelation zwischen der Expression von ETV4 und PHD2, HIF-1/2α und anderen Hypoxie-Markergenen in 282 menschlichen Geweben von Brustkrebspatientinnen.

Obwohl unsere FRET-Daten eine direkte Interaktion von HIF-α und ETV4 nahelegen, schlagen wir eher den Ansatz eines trimeren Komplexes bestehend aus HIF:p300/CBP:ETV4 vor. Bei einer tiefgehenden HIF-1α Domänenkartierung fanden wir heraus, dass die hypoxieinduzierte HIF-1α:ETV4 Interaktion hauptsächlich über die C-terminale Aktivierungsdomäne stattfindet. Darüber hinaus wurde durch die Überexpression von CBP/p300-interagierenem Transaktivator 2 (CITED2), ein Konkurrent von HIF um die p300/CBP-Interaktionsstelle, der ETV4:HIF Komplex zerstört. Dieses Resultat unterstreicht die Bedeutung von p300/CBP in der Komplexbildung. Durch die Verarmung des „HIF-inhibierenden Faktors“ (FIH) wurde eine deregulierte Bindung von HIF an p300/CBP provoziert, die den Verlust der sauerstoffabhängigen Interaktion von HIF und ETV4 zur Folge hatte. Zusammengefasst beweisen diese Experimente die Zusammenarbeit von HIF-1α und p300/CBP bei der ETV4 Bindung.

Neuste Daten weisen darauf hin, dass ETV4-Proteinlevel unter hypoxischen Bedingungen und bei PHD2-Knockdown in der Normoxie erhöht sind, während mRNA-Level sich unverändert zeigten. ETV4 Proteinlevel waren ebenfalls erhöht, wenn Zellen mit einem PHD-Inhibitor behandelt wurden.

Zusammenfassend demonstrierte diese Arbeit, dass ein synthetischer Transaktivierungsansatz unbekannte Wechselwirkungen von transkriptionellen Pfaden aufdecken kann, die zudem klinische Bedeutung bei unterschiedlichen Krebsarten haben.

1 Introduction

1.1 Hypoxia

One can survive without food for 3 weeks, without water 3 days, without warmth 3 hours, but only 3 minutes without oxygen[1]. Although these values are not exact, oxygen is unquestionably essential for mammals. Hypoxia, the undersupply of oxygen, occurs on different levels. An adaptation to hypoxia is already provoked by exposing the body to altitudes higher than 3000 m[2,3]. Physiological processes like an increased ventilation and cardiac output compensate for the decreased partial inspiratory oxygen pressure (pO_2). Later during acclimatization, oxygen transportation capacity is increased through the hormone erythropoietin (EPO), by stimulating the erythroid progenitor cells in the bone marrow.
Most of Bolivian, Tibetian and Ethiopian subjects living in high altitude and thus, constantly exposed to low pO_2 show chronically elevated hematocrit (about 10%)[4]. The undersupply of oxygen also happens within a human body. Without exposing to high altitude certain tissues can become hypoxic. This might happen when the lung function is impaired or the circulation is blocked during asthma, embolism or ischemia, respectively. Another pathophysiologic hypoxic environment is a solid tumor. Common for all solide tumors is a switch in cellular metabolism to prevent the tissue from death.

1.1.1 The hypoxia-inducible factor (HIF)

It took long to link EPO to the hypoxia-inducible factor (HIF). EPO is expressed in the fetal liver and later shifts its expression to the adult kidney. HIF was only identified in 1991 when Semenza *et al.* found a 50 nucleotide long *cis*-acting element in the *EPO* gene[5,6]. It was shown that hypoxia triggers an activation of the EPO gene in the hepatoma cell line Hep3B[6]. Also, a binding activity to the EPO-enhancer region could be revealed, which was enhanced when cells were exposed to hypoxia and was reduced when treated with the translational inhibitor cycloheximide (CHX)[7]. Four years later, in 1995, Wang and Semenza purified and identified this hypoxic DNA

binding activity[8]. It was found to be a heterodimeric protein complex of two subunits: An α-subunit (HIF-α) and a β-subunit (HIF-β). They named this transcriptional complex "HIF-1". HIF-1β is also known as aryl hydrocarbon receptor nuclear translocator (ARNT) and was previously described to heterodimerize with the aryl hydrocarbon receptor (AhR) to mediate xenobiotic responses[9].Both heterodimerization partners of HIF-1β - HIF-1α and AhR - are basic helix-loop-helix (bHLH) transcription factors belonging to the PAS subfamily (period circadian protein (PER), ARNT and single-minded protein (SIM)). Three paralogues of the HIF-α subunit are known: HIF-1α encoded by the *HIF1A* gene; HIF-2α by *endothelial PAS domain 1* (*EPAS1*); and HIF-3α by *HIF3A*. Furthermore, different β-subunits are discussed. Besides the well-studied HIF-1β (*ARNT*)[9] subunit, there are also few studies available concerning the potential interaction with HIF-2β (*ARNT2*)[10,11] or HIF-3β (*ARNT3*)[12] as well as ARNT Like (*ARNTL*)[13] and ARNT Like 2 (*ARNTL2*)[14,15] subunits.

1.1.2 Genetic ablation of HIF

Hif1a-deficient mice show a lethal phenotype by embryonic day (E) 11. This is caused by a developmental arrest of Hif1a-/- embryos that results in a defective neural tube, cardiac and vascular malformations, hypoplastic pharyngeal arches and cell death within the cephalic mesenchyme[16–18].

In contrary, *Hif2a*-knockout results in a set of different phenotypes that are eventually fatal. Prenatal death (E12.5-16.5) is probably due to vascular disorganization and an impaired catecholamine production leading to bradycardia, an improper cardiac function and circulatory failure[19,20]. Neonatal death, however, is probably caused by an insufficient production of surfactant provoking the neonatal respiratory distress syndrome[21]. Conversely, viable *Hif2a*-deficient mice have been reported by Scortegagna *et al.* using 129S6/SvEvTac mice to have a multitude of impairments such as syndromes of multiple-organ pathology (retinopathy, hepatic steatosis, cardiac hypertrophy, skeletal myopathy, etc.), biochemical abnormalities (hypoglycemia, lactic acidosis, etc.) and altered gene expression patterns[22].

So far, a *Hif3a*-deficient mouse model has not been reported yet. Also *Hif1b*-knockout mice show embryonic lethality *in utero* between E9.5-10.5[23].

Developmental abnormalities include amongst others, neural tube closure defects and forebrain hypoplasia. Prenatal lethality appears to be due to a defective composition of the placenta[23].

1.1.3 Expression pattern of HIF-α subunits

Amongst the HIF-α subunits, HIF-1α is the most ubiquitously expressed isoform. HIF-1α is expressed in different tissues and organs, whereas HIF-2α and HIF-3α are more restrictively expressed. Although HIF-1α and HIF-2α share 48% of sequence similarity, HIF-2α is mainly found in embryonic and adult endothelium. This is already suggested by HIF-2α's synonymous name EPAS-1[24].

The human *HIF3A* gene expresses 6 alternative splicing variants (HIF-3α1-6) and the full length mRNA expressed in the kidney is suggested to be HIF-3α1[25]. The splice variant HIF-3α4, also known as inhibitory PAS domain protein (IPAS), was shown to be expressed predominantly in the eye and at lower levels in the cerebellum and the cerebrum, indicating a tissue-restricted expression pattern of IPAS mRNA[26]. It was reported that IPAS might inhibit VEGF expression in murine cornea by HIF-1α binding and thus prevents a HIF-1 formation and activation of target genes that lead to a negative regulation of angiogenesis and maintenance of an avascular phenotype[27]. Complex formation of IPAS with HIF-2α was demonstrated to interfere with the expression of HIF-2α downstream targets and tumor formation[28].

1.1.4 Composition of the HIF complex

The heterodimeric transcription factor HIF contains a highly regulated α-subunit. The N-terminus of the α-subunit shows two important domains (fig. 1). First, a basic helix-loop-helix (bHLH) domain that is responsible for the specific binding to a core DNA-consensus sequence and heterodimer formation. In this work, following nomenclature is used: the HIF DNA binding site (HBS) (5'-RCGTG-3') lies inside the hypoxia response element (HRE) and represents the core region of the HRE being situated in HIF target genes[9,29,30]. The HRE describes here the minimal *cis*-regulatory

element needed for hypoxic induction of gene transcription. The HBS is necessary but does not guarantee full hypoxic gene activation. Consequently, the HBS represents the minimal DNA sequence for HIF interaction, but a fully functional HRE typically includes also neighboring DNA binding sites for additional (co-)transcription factors[31–33]. The second domain, called PAS, is subdivided into PAS A and PAS B and mediates HIF-1β dimer formation.

Interestingly, HIF-α subunits have two transactivation domains (TAD): a carboxy-terminal TAD (CTAD) and an amino-terminal TAD (NTAD). The latter overlaps with the oxygen-dependent degradation domain (ODDD) that is required for the stability regulation of the HIF-α subunits[34]. Importantly, an active nuclear localization signal can be found in the carboxy -terminal end of HIF-1α and HIF-2α[35]. The suggested amino-terminal NLS was shown not to be effective[35]. In contrast to HIF-1α and HIF-2α, HIF-3α1 although full length protein, does not contain a CTAD.

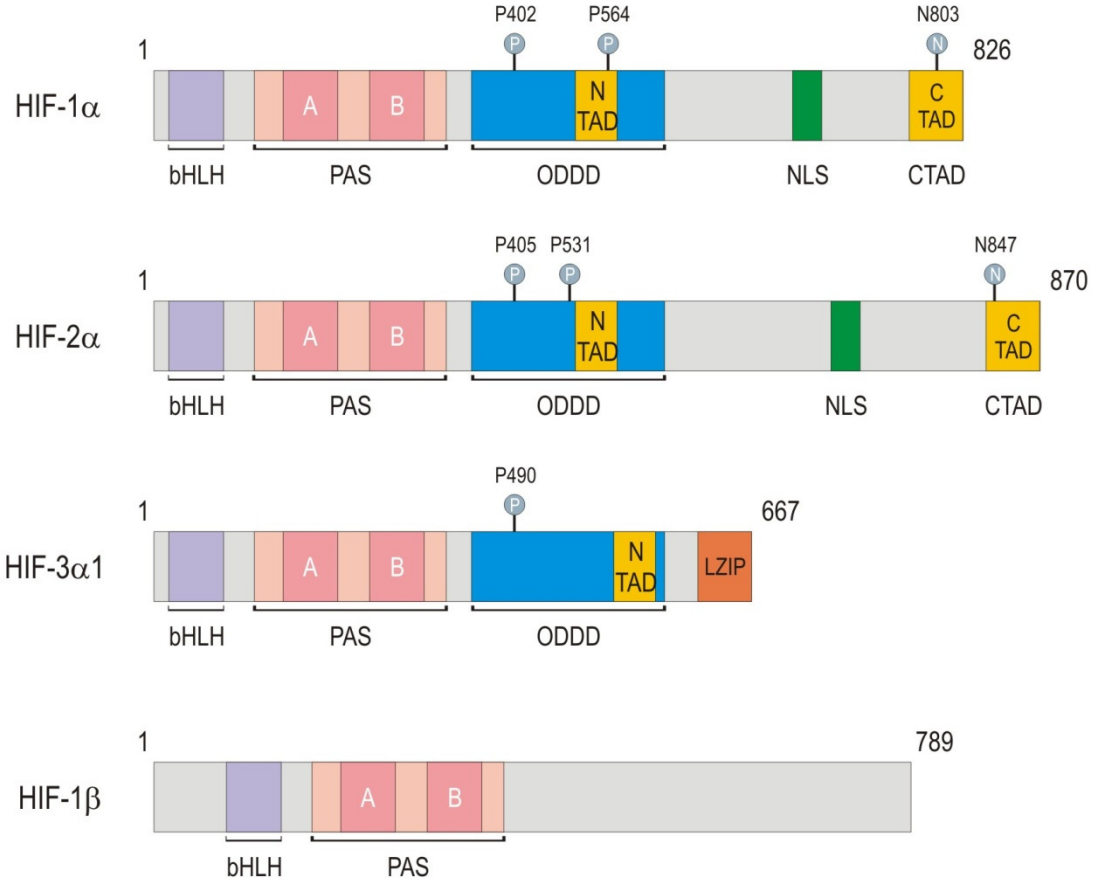

Figure 1. Domain structure of HIF-α subunits and HIF-1β

bHLH - basic helix-loop-helix; PAS - PER-ARNT-SIM (A and B), ODDD - oxygen dependent degradation domain, NTAD - Amino-terminus transactivation domain; CTAD - carboxy-terminus transactivation domain; NLS - nuclear localization sequence, LZIP - leucine zipper

1.1.5 O_2-dependent regulation of HIF-α protein stability and transactivation activity

HIF-α protein levels are firmly regulated by oxygen, whereas HIF-α mRNA levels remain unaffected. The rapid degradation of the HIF-α subunits via the ubiquitin- 26S proteasome pathway in normoxic conditions results in a half-life of less than 5 minutes. This fast degradation of the HIF-α subunit is independent of its sub-cellular localization. Therefore, the HIF-1α entry into the nucleus is not a key event that controls its stability[36]. Instead, the degradation process under normoxic conditions is mediated through the hydroxylation of distinct prolines in the ODD by prolyl-4-hydroxylase-domain containing proteins (PHDs) (fig. 2). The oxygen-dependent hydroxylation by PHDs leads to the subsequent binding of the von Hippel-Lindau protein (pVHL) tumor suppressor[37–40].

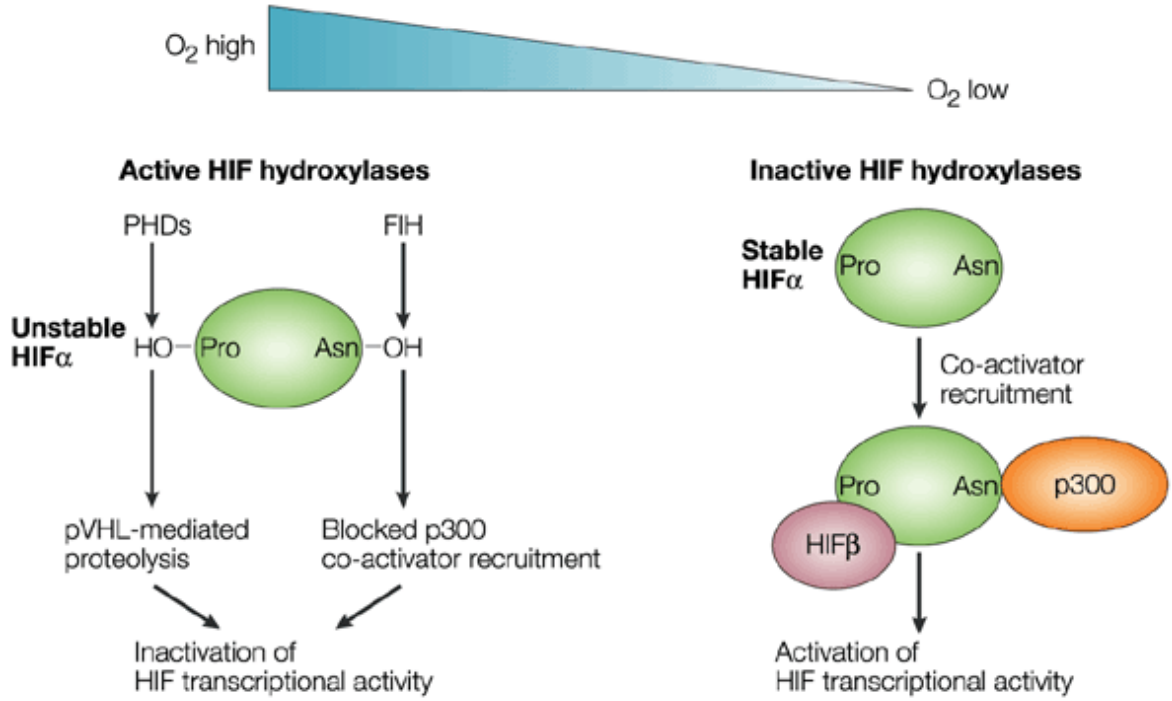

Figure 2. Scheme of oxygen sensing by PHDs[41]

Under high pO_2, HIF hydroxylases are active and destabilize the HIF-α subunits through the hydroxylation of distinct prolines by PHDs and of asparagines by factor inhibiting HIF (FIH). The prolyl-hydroxylation mediates the proteolysis via pVHL. The asparagine hydroxylation inhibits the recruitment of the co-activator p300. Both hydroxylation reactions lead to the inactivation of HIF transcriptional activity. Under low pO_2, the hydroxylation reactions are inhibited. The HIF-α subunits are no longer degraded and recruit the transcriptional co-activator p300. This leads to a powerful transcriptional activation of HIF.

VHL is one component in the complex with elongin B, elongin C, cullin-2 and its main function is suggested to be the E3 ubiquitin ligase activity for specific degradation labeling. The loss of pVHL leads to a rare inherited cancer syndrome known as VHL disease. This condition predestinates to a variety of malignant and benign tumors of the eye, brain and spinal cord (hemangioblastoma); kidney (clear cell carcinoma), pancreas, and adrenal glands (pheochromocytoma), caused by an abnormal accumulation of HIF-α subunits in normoxia[39].

Another regulation of the HIF-α subunit protein stability might be the acetylation of the amino acid (aa) 532 by the murine acetyltransferase arrest-defective-1 (ARD1) protein. The mARD1-mediated acetylation might enhance an interaction of HIF-1α with pVHL and HIF-1α ubiquitination. This suggests that the acetylation of HIF-1α by mARD1 and the support of the complex formation of HIF-1α with pVHL is critical for accelerated proteasomal degradation[42].

In contrast to this report, data were published showing no evidence for a HIF-1α destabilization through acetylation, leading to an ongoing debate on the role of acetylation-mediated destabilization of HIF-α[43–45].

These findings led to a model that is now widely accepted for regulation of HIF-α subunits: in decreased O_2 levels the substrate for the hydroxylation reaction by the PHDs, namely oxygen, is missing. The hydroxylation reaction is inhibited and the HIF-α subunits are no longer marked for their degradation. This leads to an accumulation of HIF-α subunits in the cytoplasm. They translocate into the nucleus, where they heterodimerize with the constitutively expressed HIF-β subunit and form the transcription factor HIF. HIF binds to the conserved DNA consensus sequence 5'-$^{G}/_{A}$CGTG-3' (HREs) situated mostly in promoter sites[30]. With its transcriptional activation HIF induces the gene expression of target genes promoting the adaptation to low oxygen concentrations on cellular and systemic levels. The adaptation is mediated by changes in gene expression that lead to an enhancement of oxygen delivery and / or promote survival in low oxygen conditions. The list of roughly 100 known HIF target genes comprises angiogenesis, erythropoiesis and ventilation supporting gene products, i.e. vascular endothelial growth factor (VEGF), transferrin (TF) and heme oxygenase-1 (HO-1); as well as glycolysis and autophagy supporting mechanisms as glycolytic enzymes (aldolase A, enolase 1, lactate dehydrogenase A)

and glucose transporters (i.e. Glut-1)[46–52]. Interestingly, PHD2 and PHD3 are HIF target genes as well. Thus, PHD2 and PHD3 expression is increased and consequently compensate for the decreased enzyme activity. This limits the HIF response under hypoxic conditions and leads to a rapid degradation of HIF-α subunits following reoxygenation[53–55].

Additionally, the transcriptional activity of HIF is tuned by the factor inhibiting HIF (FIH)[56]. FIH was reported to hydroxylate an asparagine (N) residue and subsequently hinder the association with p300/CBP via the cysteine/histidine rich (CH)-1 domain. Eventually, this results in the prevention of the formation a transcriptional active HIF complex[57]. The location of the essential asparagine residue hydroxylated by FIH in the CTAD differ inbetween the HIF-α isoforms. In the HIF-1α subunit the distinct amino acid N803 is hydroxylated, whereas in HIF-2α it is the asparagines residue 847.

In summary, it could be shown that an intracellular O_2-sensor system exists and that the O_2 tension is connected with HIF-α protein stability and hence the transcriptional activity of genes. The system can be tuned by a further decrease of pO_2 that leads to an inactivation of FIH and the disinhibition of the recruitment of co-activators such as p300/CBP, steroid receptor coactivator 1 (SRC-1), transcription intermidiary factor 2 (TIF2) and Redox factor-1 (Ref-1)[58–61].

1.1.6 O_2-independent regulation of HIF transactivation

The HIF activation and stabilization can also be regulated by oxygen-independent HIF-α protein synthesis or posttranslational modifications other than the previously described oxygen-dependent hydroxylation of proline and asparagine residues of the HIF-α subunit. Many interplaying processes are discussed in the field. For example, the S-nitrosylation of the cysteine residue 800 (C800) has been reported to activate the interaction between stabilized HIF-1α and the co-activator CBP/p300, eventually stimulating transactivation of the HIF-complex[62].
Another mechanism shown to increase the transcriptional activity of HIF is the phosphorylation of threonine 796 (T796) and of the serine residues 641 and 643

(S641, S643) by the mitogen-activated protein kinases (MAPKs)[63–65]. The latter seems to control HIF-1α mediated translocation to the nucleus and as a consequence the transcriptional activity of the HIF complex[66].

Lately, also SUMOylation, meaning the attachment or detachment of small ubiquitin-related modifier (SUMO) is discussed. However, conflicting results have been reported leading to an increase or a decrease of HIF-α stability[67–69]. SUMOylated HIF-α is reported to be recognized by VHL and additionally, mice lacking the SUMO-specific protease 1 (SENP1) develop fetal anemia, insufficient EPO production and undergo prenatal death (E13-E15)[67]. Conversely, SUMO1 was reported to be itself hypoxia inducible suggesting that HIF is stabilized in hypoxic conditions and thus, influences HIF protein stability and transcriptional activity[68–70].

Along with the aforementioned modifications heat shock protein 90 (Hsp90) has been implicated to promote HIF stability in a VHL-dependent manner, since Hsp90-inhibitors apparently promote HIF-α degradation[71,72].

Recent studies suggest that also the transcript levels of HIF-1α are increased by NFκB upon stimulation through reactive oxygen species (ROS) and lipopolysaccharide (LPS), probably via a conserved κB site in the human and murine *HIF1a* gene[73–78].

Together with the mentioned modifications, HIF has been shown to be influenced in its protein synthesis rate by growth factors (IGF-1, IGF-2), cytokines (TNFα, IL-1β) and Insulin[79–86]. Often those stimuli are interlinked with cell growth that is in turned associated with an increased oxygen demand. The cells adjust to the expected rise in oxygen consumption by inducing cellular signaling pathways (often kinase-related) and ROS (partially in combination). For example, stimulation of the PI3K pathway by insulin-like growth factor 1 (IGF-1) leads to an overall increase in protein translation, a saturation of the degradation machinery and eventually in an oxygen-independent accumulation of HIF-α[83,87].

1.2 Prolyl-4-hydroxylase domain containing proteins (PHDs)

1.2.1 Discovery and characterization of PHDs

It needed about a decade after the discovery of HIF to find a protein that is capable to sense changes in oxygen availability and converting this signal to a shift in HIF-α stability. It was known that in the presence of oxygen HIF-α subunit is recognized by an E3 ubiquitin ligase containing the pVHL tumor suppressor protein and is targeted for proteasomal degradation[37]. In 2001, a conserved family of HIF prolyl-4-hydoxylase (PHD or alternatively HIF prolyl hydroxylase (HPH)) enzymes was identified which was responsible for this posttranslational modification of HIF-α[37,88–92]. The first identification of a unique PHD was in *Caenorhabditis elegans* and named egg-laying abnormal-9 (EGL-9)[89]. In mammals three different isoforms were discovered: PHD1 (HPH3, EGLN2), PHD2 (HPH2, EGLN1) and PHD3 (HPH1, EGLN3). Alternative splicing forms have been reported for PHD2 and PHD3, but not for PHD1[93,94]. The proteins differ in size, intracellular localization and tissue distribution[95]. In contrast to PHD2 and PHD3, PHD1 also exists in a shorter fully functional isoform generated from alternative translational initiation[96]. The two critical prolines of the ODD are both independent and non-redundant hydroxylation targets and interact therefore separately with the pVHL complex[97]. Interestingly, under *in vitro* conditions the different PHD isoforms also have distinguishable preferences to hydroxylate the two target prolines in the HIF-ODD[98,99]. In conclusion, PHDs are considered to be cellular oxygen sensors since they were demonstrated to modulate the HIF-α stability in an oxygen-dependent mode through proline hydroxylation[88,92].

Lately, a fourth PHD-related protein (PH-4) was discovered[100,101]. PH-4 was reported to be able to hydroxylate the two critical prolyl residues of the HIF-1α subunit, but unlike PHDs which localize to the cytoplasm and nucleus - was associated with the endoplasmic reticulum (ER). However, the functional relevance of PH-4 for the hypoxia-induced pathway is still elusive.

1.2.2 The enzymatic reaction

HIF-prolyl-hydroxylases are members of the iron (Fe^{2+}) and 2-oxoglutarate (2-OG)-dependent family of dioxygenases, similar to collagen prolyl-hydroxylases. Members of this family need molecular oxygen, Fe^{2+} and 2-OG for their enzymatic reaction. During the catalyzed reaction one oxygen atom is used in a decarboxylation reaction converting 2-OG to succinate and providing the second oxygen atom for the hydroxylation of the prolyl residue (fig. 3)[102]. Proline hydroxylation is an irreversible reaction.

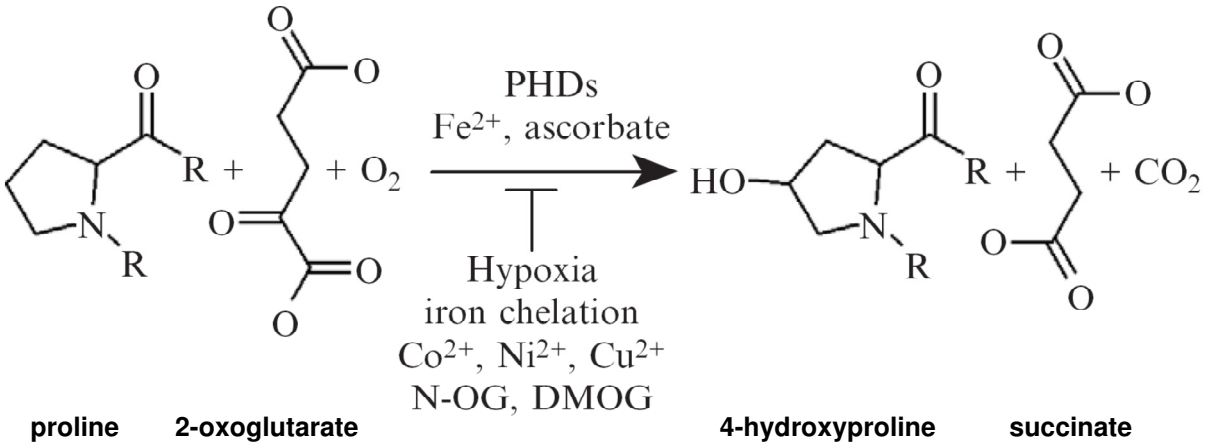

Figure 3. The enzymatic reaction of proline hydroxylation catalyzed by HIF-prolyl-4-hydroxylases[102].

Essential co-factors of the reaction are ferrous iron (Fe^{2+}) and ascorbate, whereas hypoxia, iron chelators, transition metals (Co^{2+}, Ni^{2+}, Cu^{2+}), dimethyloxalylglycine (DMOG) and N-oxalylglycine (N-OG) are inhibiting the reaction.

Essential co-factors in this reaction are Fe^{2+} (ferrous iron) and ascorbate (vitamin C). It is speculated that ascorbate might have a protective role against oxidation of the Fe^{2+} incorporated in the active center of the PHD or for the enzyme itself[103,104]. However, very recently ascorbate was found to be not required for oxygen sensing in ascorbate-deprived Gulo(-/-) knockout mice since they responded normal to inspiratory hypoxia[105]. Also, hypoxic HIF induction was essentially normal under serum- and ascorbate-free cell culture conditions and glutathione was demonstrated to be able to fully substitute for the vitamin C requirement of all three PHD isoforms *in vitro*[105].

Two distinct prolines located in the HIF-1α ODDD are hydroxylated by PHDs, namely Pro402 and Pro564. The PHDs seem to have a preferential ranking for hydroxylating those prolines. The proline residue 564 seems to be hydroxylated by all PHD isoforms, whereas Pro402 is only hydroxylated by PHD1 and PHD2 *in vitro*[89]. Nevertheless, by overexpressing PHDs in cells, all PHD isoforms could hydroxylate both proline residues[99].

It could be shown that the two prolines have independent yet interactive roles in the regulation of HIF-1α protein turnover and that under normoxic conditions proline (P) 564 is hydroxylated prior to P402. With decreasing pO_2, the hydroxylation of P402 stops earlier then the hydroxylation of P564 and the inhibition of P402 hydroxylation is sufficient to induce HIF-α stabilization in hypoxia, while hydroxylation of P564 was still observed[99]. So far the "motif" for proline residues that are considered as targets for hydroxylation is L-X-X-L-A-P (X stands for any amino acid)[106]. However, only the proline in this motif is indispensable for recognition, which makes the relevance of the defined pattern questionable[106–108].

1.2.3 Modulation of PHD activity and co-factors

The hydroxylation capacity of PHDs is influenced by many factors. The availability of oxygen, 2-oxoglutarate, Fe^{2+} and ascorbate together with the interplay of small molecules regulate the activity of PHDs and consequently the abundance of HIF and its transcriptional activity.

Oxygen

Oxygen is the most important factor for the hydroxylation reaction. In an *in vitro* hydroxylation-coupled decarboxylation assay the Michaelis-Menten (K_m) constant of PHDs for O_2 was determined to be in the range of 230-250 μM[109]. However, these K_m values are still above the tissue O_2 concentration of 10-30 μM delivering the oxygen necessary for the hydroxylation reaction[110]. This is a cornerstone feature of the PHDs because the uneffective hydroxylation reaction permits a fine-tuned response to small oxygen changes that "translate" later into different degrees of HIF-α stabilization. Intriguingly, the K_m for oxygen of FIH was determined to be even lower, namely 90 μM, meaning that FIH activity is maintained under hypoxic conditions

limiting the transcriptional power of the accumulated HIF[111]. This decreased K_m with respect to the PHD enzymes suggests that FIH is still functional at reduced O_2 concentrations where HIF-α protein is already accumulating.

In spite of this, more recent studies employing longer and thus also more physiological HIF-α peptides indicate that the oxygen K_m of PHDs are rather in the range of 100 μM[112,113]. It was demonstrated that the determination of K_m values is influenced by the purity and origin (insect cells, bacteria) of the enzymes as well as dependent on the length of the substrate (the longer, the more physiological e.g. HIF-fragment) used for oxygen consumption assays or *in vitro* hydroxylation studies[112–114].

Tricarboxylic acid cycle and metabolic intermediates

Another cosubstrate for PHDs is 2-OG (2-oxoglutarate) coming from the tricarboxylic acid cycle (TCA). The K_m constant of PHDs for 2-OG was determined to be around 60 μM[115]. Binding of 2-OG to the enzyme is required for normal turnover of the enzyme, so that 2-OG analogs inhibiting enzyme activity by binding to the 2-OG pocket. PHDs were also reported to be reduced by glucose metabolites such as pyruvate, citrate, iso-citrate, succinate, fumarate, malate and oxaloacetate supporting HIF-α accumulation[104,116–120].

Iron and divalent metals

The cofactor Fe^{2+} is tightly bound by PHDs. The K_m values for PHD1 and PHD2 were suggested to be around 30 nM, whereas PHD3 shows a K_m of about 100 nM[121]. As a consequence, iron chelators have impact on the HIF pathway. Hirslä *et al.* further demonstrated that PHDs are affected in their activity by divalent metals, such as Cd^{2+}, Ni^{2+}, Mg^{2+}, Mn^{2+} and Co^{2+}, which converts to the activation of the HIF system[121]. Mechanistically, divalent metals are suggested to operate either by replacement or oxidation of PHD bound Fe^{2+}. Cu^{2+} was shown to cause HIF-1α accumulation and reporter gene activation in normoxia[122,123]. Nytko *et al.* explain that this occurs in the presence of oxygen through a strong Cu^{2+}-catalysed oxidation of ascorbate[104]. The strong inhibitory effects of Co^{2+} were suggested to originate from direct binding of Co^{2+} to HIF-1α causing an inhibition of HIF-1α protein degradation

by the VHL ubiquitin E3 ligase complex[124]. Additionally, Ca^{2+} interferes with the PHD2 activity and Ca^{2+} chelation lead to PHD2 inhibition[125].

Nitric oxide

Nitric oxide (NO) showed modulating effects on PHD activities. NO derived from NO donors or inducible NO synthase (iNOS) directly inhibits PHD function and thus leads to HIF-α stabilization[126–128]. The inhibition of PHD might be derived from iron oxidation in the catalytic center. Interestingly, endothelial NOS (eNOS) is a HIF target gene that could provide an additional layer of feedback regulation to the system[114].

On the other hand, NO has shown opposing effects by decreasing hypoxia induced HIF-1α levels. This ambivalent response to NO treatment could also be explained by a chronological order of the events: in the early phase, when NO is present, PHD activity is inhibited and thus HIF stabilized. In a later phase HIF-dependent *de novo* PHD2 synthesis is increased. Additionally, mitochondrial oxygen consumption is inhibited which results in increased intracellular oxygen availability. The rise of both PHD2 and oxygen availability leads to a decreased NO-dependent HIF-1α stabilization[129–131].

Reactive oxygen species and antioxidants

Production of reactive oxygen species (ROS) were shown to be increased in hypoxia presumably due to an inhibition of the respiratory chain[132–134]. Additionally, cells deficient in mitochondrial DNA have been demonstrated not to activate HIF-target gene expression[132,135]. That implies again an involvement of mitochondria in the oxygen sensing pathway. Of note, this finding could not be confirmed by other groups, especially in respect to the controversially discussed questions if ROS are able to be generated during hypoxia, before reoxygenation and if ROS really are implied in the regulation of HIF-α stability[136–139]. ROS inhibit the hydroxylation capacity of PHDs by oxidizing either directly amino acid residues of the enzyme or indirectly oxidizing cofactors (Fe^{2+}, ascorbate) resulting in an inactivation or cellular depletion. The antioxidant ascorbate as has been shown to prevent Fe^{2+} oxidation in the catalytic center of PHDs or for the enzyme itself and thus support *in vitro* hydroxylation of HIF-α ODD fusion protein constructs[103,104,126,140]. The K_m values of PHDs for ascorbate is between 140-180 µM and Nytko *et al.* showed that ascorbate affects oxygen sensing neither *in vitro* nor in an ascorbate-deprived Gulo(-/-) mouse model *in vivo*[105,115,141].

However, cells deleted for the transcription factor JunD, belonging to the AP1-family members and involved in the oxidative stress defense, have been shown to inhibit prolyl hydroxylation by enhancing the hydrogen peroxide (H_2O_2) metabolism, followed by iron oxidation and HIF-α accumulation, eventually resulting in the activation of HIF-target genes[142]. Further, the oncoprotein mucin 1 (MUC1) was reported - lacking detailed mechanistic understanding - to be able to suppress hypoxia-induced ROS accumulation and to induce PHD3 expression which results in an attenuated HIF transcriptional activity[143–145].

1.2.4 Subcellular and tissue distribution of PHDs

In cells, PHDs contribute differentially to the regulation of HIF based on their abundance[98]. PHDs are differently expressed on both cellular and systemic levels. On the cellular level fluorescent fusion proteins revealed that PHD1 was exclusively detected in the nucleus, PHD2 was located in the cytoplasm, and PHD3 was homogeneously distributed in cytoplasm and nucleus[106,146]. On the other hand, endogenous PHD2 and PHD3 were found in cytoplasm and nucleus in rat hepatocytes[147]. Interestingly, in this approach PHD1 was found in the nucleus during normoxic conditions, but shuttled to the cytoplasm in hypoxia and back to the nucleus following reoxygenation.

On the tissue level, PHD2 is the most abundant one, ubiquitously expressed in most tissues, whereas PHD1 is strongly expressed in testis and PHD3 shows highest expression in the heart[100,148,149].

1.2.5 Genetic ablation of PHDs

The knockout of PHD2, but not PHD1 or PHD3, in mice leads to embryonic lethality in E12.5-14.5 due to placental and heart defects[150]. Conditional PHD2 knockout mice show an increased erythropoiesis and angiogenesis through HIF stabilization and the downstream targets EPO and serum VEGF-a[151]. Eventually, mice with conditional PHD2-inactivation (mated with beta-actin-Cre-ER mice) die from venous congestion and dilated cardiomyopathy[152]. Interestingly, a study in human subjects confirmed the

critical role of PHD2 in human physiology. Mutations in the human *PHD2* gene have been demonstrated to cause erythrocytosis, a familial hereditary disease characterized by the excessive production of erythrocytes[153,154].

The genetic ablation of PHD3 results in an abnormal sympathoadrenal development and systemic hypotension[155]. The authors of the above mentioned study suggest that functional PHD3 is essential for the correct anatomical and physiological status of the organisms and the PHD3-HIF-2α pathway is not redundant[155]. A combined knockout of PHD3 and PHD2 aggravate HIF activation (compared to mice lacking PHD2 alone). These mice have shown symptoms such as hepatic steatosis, dilated cardiomyopathy and premature mortality[156]. The phenotype resembles more pVHL-deficient mice[156,157].

PHD1-deficient mice show a shift in glucose metabolism from aerobic oxidation to anaerobic glycolysis and render the muscle fibers tolerant to hypoxia through lowered oxygen consumption[158]. As a consequence, the mice have been demonstrated to be protected against ischemic injury in heart and liver, as well as colitis[158–160].

A combined PHD1 and PHD3 knockout led to moderate erythrocytosis and HIF-2α accumulated in the liver resulting in an increased EPO expression in adult livers and as a consequence a slight increase of the hematocrit, hemoglobin concentration and red blood cell amount[161].

1.2.6 Regulation of PHD expression

PHD1 gene expression is induced by estradiol[162]. PHD2 expression has been shown to be regulated by TGF-β by decreasing PHD2 mRNA and protein levels and consequently inducing HIF-1 accumulation and transcriptional activity[163].

Interestingly, PHD2 and PHD3, but not PHD1, are direct HIF target genes, resulting in up-regulated mRNA expression levels under hypoxic conditions[30]. The increased abundance of the two oxygen-dependent regulated PHDs partially compensates for the decreased activity of the hydroxylases in hypoxia. As a consequence HIF-α accumulation is restricted and the hypoxic response is limited[164]. Further, in case of reoxygenation after long-term hypoxia, this negative regulatory mechanism causes

an accumulation of PHD2 and PHD3 leading to an accelerated degradation of HIF-α (fig. 4)[165–168].

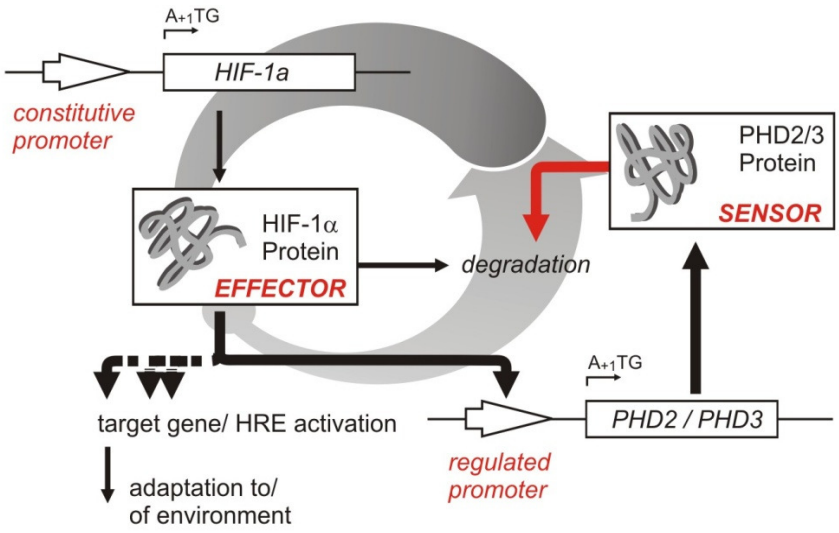

Figure 4. Illustration of HIF-PHD2/3 negative feedback loop.

The interplay of the HIF-α subunit and the PHDs can be regarded as a classical negative feedback loop. HIF-1α takes the role of the main effector and PHD2/PHD3 of O_2-sensors, initiating HIF-α degradation. If high levels of the constitutively expressed HIF-1α accumulate, HIF will bind to the promoter of PHD2 and PHD3. This results in an increased expression of PHDs, which subsequently mark the HIF-1α subunit for degradation and eventually suppresses the PHD2/3 expression. This effect allows a stabilization of HIF-1α again. The feedback loop limits hypoxic signaling and accelerates HIF degradation after reoxygenation (modified; figure kindly provided by DP Stiehl).

The regulation of *EGLN1* (*PHD2*) and *EGLN3* (*PHD3*) gene is based on a functional HBS located in the corresponding genes. The regulatory HBS of PHD2 is located in a CpG island situated in the *PHD2* promoter immediately upstream of the translational start site (fig. 5)[169]. In contrast, an HBS was reported to be located in a conserved region of the first intron of the *EGLN3* gene 12 kb downstream of the transcription initiation site[55]. However, the hypoxic regulation of PHDs transcription, other than through the transcription factor HIF, is not yet fully understood.

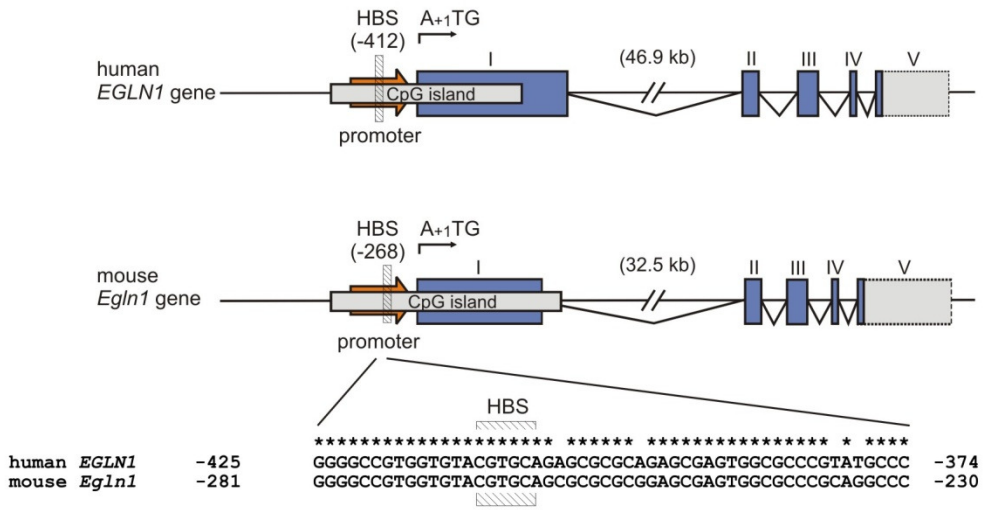

Figure 5. The *EGLN1* / *Egln1* (*PHD2*) gene in the human and murine genome.
The human *PHD2* promoter region was predicted based on the location of a CpG-island approximately 0.5 kb upstream of the translational start site and had been proven experimentally to be functional[170]. The murine and human *EGLN1* (*PHD2*) gene sequences were aligned. However, expressed sequence tag (EST) alignments did not lead to a precise mapping of the transcriptional start site. Prediction analysis of potential transcription factor binding sites for the human and murine *PHD2* gene was performed and the HBS was found to be conserved between these species (modified from E Metzen *et al.*[169]).

1.2.7 Other regulatory mechanisms of PHD expression and degradation

PHDs are not only differently expressed but also degraded via different pathways. PHD1 and PHD3 have been reported to be Siah1/2 targets, an E3 ubiquitin ligase, and consequently degraded via the proteasome. In contrast, PHD2 protein stability is regulated by an ubiquitin-independent proteasomal pathway involving the interaction with the peptidyl prolyl cis/trans isomerase FK506-binding protein (FKBP38) as adaptor protein. Furthermore, FKBP38 mediates proteasomal interaction leading to degradation of FKBP38-bound PHD2 whereas cytosolic PHD2 is stable and able to function as an active prolyl-4-hydroxylase[171,172].

1.2.8 Interactors and putative HIF-α hydroxylation substrates of PHDs

PHDs are not only regulated by their expression levels and activity, but their function might also be influenced through the interaction with different proteins. Characterized PHD interactors are listed in table 1[95]. Some of these interactors are potentially hydroxylated by PHDs.

interactor	action	PHD
Siah2[173]	inhibits	PHD1/3
FKBP38[171]	inhibits	PHD2
TriC[174]	activates	PHD3
OS-9[175]	activates	PHD2/3
AKAP[176]	activates	PHD2
Morg1[177]	activates	PHD3
ING4[178]	inhibits	PHD2
IOP1[179]	activates	PHD2
MAGE[180]	inhibits	PHD1/3
Cdr2[181]	activates	PHD1
ATF4[182]	is inhibited	PHD3
IKKβ[183]	is inhibited	PHD1
Myogenin[184]	Is activated	PHD3
KIFBβ[185]	is activated	PHD3
Rbp1[186]	is inhibited	PHD1
PKM2[187]	is activated	PHD3

Table 1. Characterized interactors of PHD1-3.

Shading indicates putative hydroxylation through PHDs (modified from RH Wenger *et al.*[95]).

1.2.9 Relevance of PHDs in physiology and pathophysiology

Numerous diseases like anemia, ischemia and stroke, but also solid cancer development are associated with inadequate tissue oxygenation. As PHDs play an important role in HIF-α-destabilization, they are interesting targets for inhibitor treatment of different diseases.

It is known that HIFα-stabilization has a positive influence on anemia, ischemia and stroke since erythrocyte production, angiogenesis and tissue survival is supported[188]. However, a prolonged PHD-inhibitor administration might have severe side effects by promoting angiogenesis, cancer cell invasion of healthy surrounding tissue and tumorigenesis[189]. PHD inhibition was shown to be beneficial for neuronal survival and reduced brain infarct volume after transient ischemia through carotid artery occlusion[190,191]. Further, transient RNAi-mediated PHD2 knockdown showed to attenuate ischemia reperfusion injury and decreased the infarct size in the myocardial tissue[192].

Another field of PHD inhibitor application is the treatment of inflammatory bowel disease, an inflammatory condition of the gastrointestinal tract. In mouse models the progression of colitis and inflammatory markers, such as IL-1β, TNF-α and IL-12 could be attenuated by the administration of the PHD inhibitor dimethyloxalylglycine (DMOG)[193].

On the other hand the activation of PHDs may also comprise options in cancer treatments. Already at the size of a few millimeters solid tumors are insufficiently penetrated by nutrients and oxygen. Consequently, hypoxic regions develop where HIF-α is stabilized and induces the transcription of HIF downstream targets beneficial for tumor survival and growth[194]. The role of PHD2, the main cellular oxygen sensor, is controversially discussed. On the one hand, decreased PHD2 levels were shown to promote breast cancer progression together with an increase in mature blood vessels in the tumor[195,196]. On the other hand, tumors injected into PHD2 haplodeficient mice were reported to show an enhanced tumor perfusion and oxygenation by vessel normalization and inhibited tumor cell invasion[197]. This apparent divergence in the role of PHD2 in angiogenesis was attempted to be elucidated by distinguishing the origin of PHD2 deficiency: if the tumor cells themselves are deficient in PHD2 an increased angiogenic response is triggered,

whereas if the host endothelium is lacking PHD2, the blood vessels structure is normalized[198].

In conclusion, the modulation of PHD activity to eventually influence HIF-α stability seems to be an attractive option of treating various conditions. However, the precise biochemical mechanisms of HIF-α hydroxylation by PHDs as well as possible other, yet unknown, hydroxylation targets may cause severe side effects in patients.

1.3 ETS factors

Several lines of evidence point to an involvement of the E-twenty six (ETS) transcription factor family in regulating different hypoxia-inducible genes[199–202]. For example, ETS1 has been shown to up-regulate hypoxia-inducible genes such as *carbonic anhydrase 9* (*CA9*), *lysyloxidase like 2* (*LOXL2*) and *n-myc-down regulated 1* (*NDRG1*) / *calcium activated protein* (*Cap43*) in a HIF-independent manner[199]. Additionally, promoter analysis revealed putative binding sites for members of the ETS transcription factor family in all target genes that were exclusively HIF-2α dependent. These potential ETS-binding sites were in proximity to putative HREs in 90% of the cases[200].

The *ETS* gene family is a transcription factor family that can be found in all metazoans[203]. The oncogene *v-ets* was at first discovered as part of a fusion protein of a transforming retrovirus (avian erythroblastosis virus E26) and later also found as cellular gene, termed ETS-1[204–206]. Two years later the most similar ets-protein in comparison to ETS-1, named ETS-2, was identified[207]. Subsequently, the family of ETS genes enlarged enormously. Up-to-date there are at least 29 members of the ETS transcription factor family known, characterized by a conserved 85-amino acids DNA-binding domain, termed the ETS-domain. According to the sequence similarity in this ETS-domain, the position of the domain and additional conserved domains amongst the family members the ETS proteins are subdivided into approximately 12 subfamilies (ETS, ERG, ELG, ELF, ESE, ERF, PEA3, Er71, TEL, SPI, TCF and PDEF)[208,209] (fig. 6). The ETS domain has been shown to interact specifically with a 9 to 15 nucleotide DNA sequence in the consensus core sequence 5'-GGA$^{A}/_{T}$-3', termed ETS binding site (EBS)[210,211]. The EBS is found in promoters of diverse genes involved in development, proliferation, differentiation and in pathologies such as cancer[212]. However, the ETS-domain can be also involved in protein-protein interactions especially with transcriptional cofactors that guide the transcriptional activity[213,214].

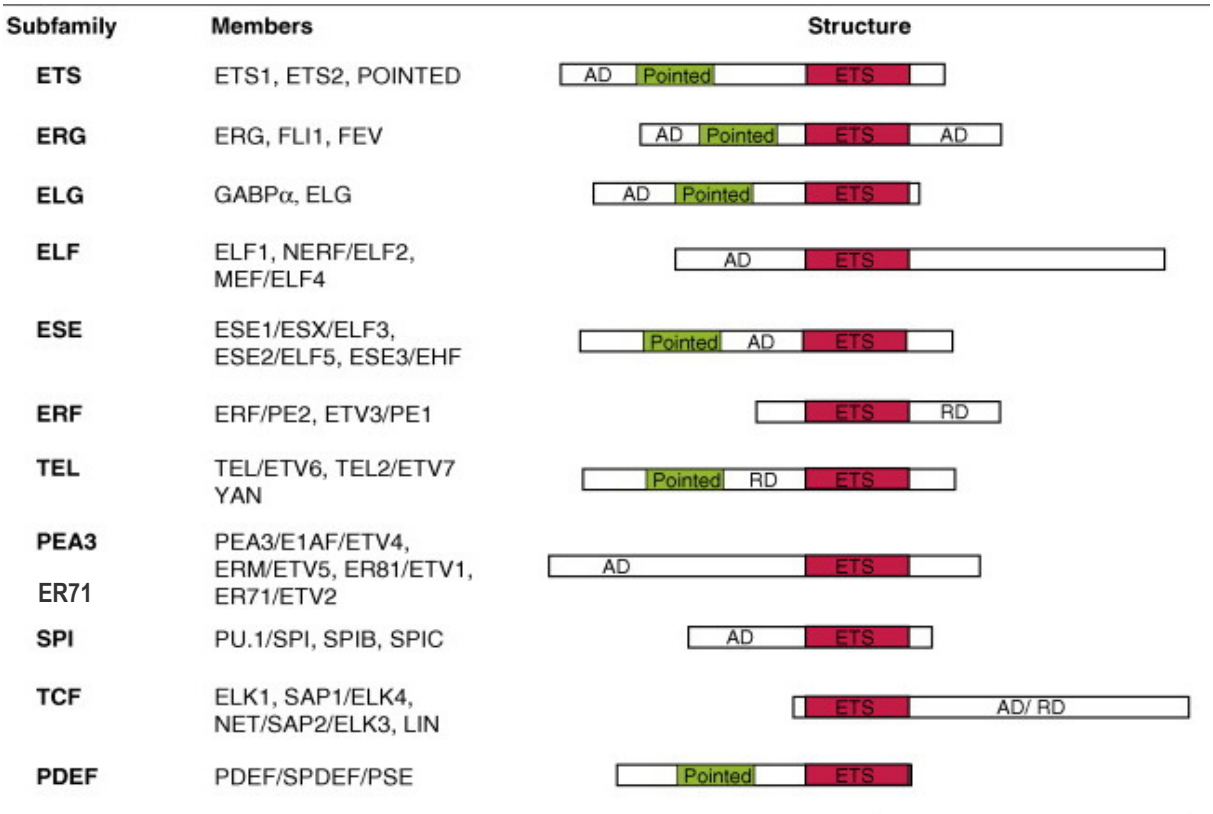

Figure 6. The 12 subfamilies of the ETS family of transcription factors and their members.
The main functional domains characteristic of members of each ETS subfamily are depicted; alternative names for each member are given (separated by a slash). Domains: AD, transcriptional activation domain; ETS, DNA binding domain; Pointed, basic helix–loop–helix pointed domain; RD, transcriptional repressor domain. Protein abbreviations: E1AF, E1A enhancer binding protein; EHF, ETS homologous factor; ELF, E74-like factor; ELG, ETS like gene; ER81, ETS related protein 81; ERF, ETS repressor factor; ERG, v-ets avian erythroblastosis virus E26 oncogene related; ERM, ETS related molecule; ESE, epithelial specific ETS; ETS, v-ets erythroblastosis virus E26 oncogene homolog; ETV, ETS variant gene; FLI1, Friend leukemia virus integration 1; FEV, fifth Ewing variant; GABP, GA repeat binding protein; LIN, abnormal cell lineage; MEF, myeloid ELF1-like factor; NERF, new ETS-related factor; PEA3, polyomavirus enhancer activator-3; PDEF, prostate derived ETS transcription factor; PSE, prostate epithelium-specific ETS; SAP, serum response factor accessory protein; SPDEF, SAM pointed domain containing ETS transcription factor; SPI, spleen focus forming virus proviral integration oncogene; TEL, translocation, Ets, leukemia; TCF, Ternary complex factor (modified from A Gutierrez-Hartmann)[215].

ETS transcription factors are usually activators of transcription, with some exceptions. For example, in humans ETS-2 repressor factor (ERF)[216]and in *Drosophila* Yan, an isoform of E74 are transcriptional repressors[217,218]. Transcriptional activation is realized by either the interaction with coactivators

through the transactivation domains, the synergy on other transcription factors, or through the direct influence with the general transcription machinery[212].

Apart from the ETS-domain the primary structure amongst the ETS transcription factors is variable. First structural studies of an ETS-DNA-binding domain of Friend leukemia virus integration 1 (FLI-1) provided insights that the ETS-domain is a variant of the winged helix-turn-helix motif[219]. Later studies with other ETS factors showed a high degree of secondary structure conservation, each containing three α-helices and four β-sheets (fig. 7)[220,221].

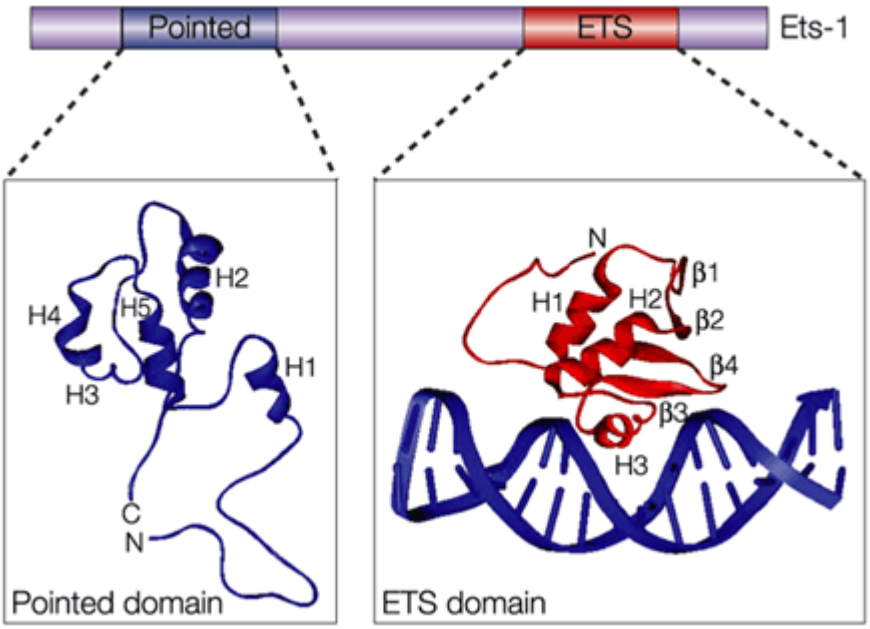

Figure 7. The location of helices (H) and β-strands (β) within the structures of the pointed domain95 (blue) and ETS domain18 (red) of ETS-1 are shown[220].

Additionally, another motif termed Pointed domain (PNT) was found in several ETS proteins[220,222]. The PNT domain contains a helix-loop-helix (HLH) domain that may support dimerization with ETS proteins[223]. Conversely the PNT domain was found to consist of 4 to 5 helices, but does not show any characteristics of a HLH motif[220,222]. However, it was found that the PNT domain of the ETS protein translocation (TEL) is able to support the formation of chimaeric kinase domain fusion proteins and thereby deregulates kinase activity and oncogenic progression in human leukemia pathologies[224,225]. Nevertheless, almost all ETS transcription factors, apart from GA repeat binding protein α (GABPα), act as monomers when they bind to DNA[226].

1.3.1 The ETS translocation variant 4 (ETV4) subfamily of ETS proteins

As described above, the family of ETS transcription factor comprises 12 subgroups. One of those subgroups is the polyomavirus enhancer activator-3 (PEA3), named after the group founding member PEA3 (also known as and later referred to as ETS translocation variant 4 (ETV4) or E1A enhancer binding protein (E1AF)). Additionally to ETV4 the subgroup consists of two other members: Er81 (ETS related protein 81, aka and from here on referred to as ETV1)[227,228] and ERM (ETS related molecule, alias and from here on referred to as ETV5)[229,230].

1.3.1.1 ETV4 subfamily protein structure and function

The three ETV4 family members share a ≥95% sequence similarity in their ETS domain. Further they show an approximately 80% similarity in their acidic domains and about 50% of the overall primary structure is similar (fig. 8)[231].

Figure 8. Domain structure of the ETV4 subfamily proteins (ETV1, ETV4, ETV5)
AD - transactivation domain; NRR - negative regulatory region (of transactivation or DNA-binding); ETS - ETS domain (adapted from Y de Launoit *et al.*[232,233])

In view of the fact that the ETV4 family members show ≥95% sequence similarity in their ETS domain that is responsible for DNA-binding, it is speculated that the ETV4 subgroup of transcription factors bind similar promoter regions with identical binding affinities.

The three ETV4 subfamily members act as transcriptional activators. Their transactivation domain is an about 40 amino acid long region of acidic residues that is suggested to be able to form α-helices that may interact directly with the general transcription factor $TAF_{II}60$[234].

However, DNA-binding and transactivation capacities of ETV4 subfamily proteins were shown also to be negatively influenced by domains surrounding the ETS DNA-binding and acidic transactivation domains, respectively[235–237]. It has been suggested that this could involve conformational changes in the N- and C-terminal helices flanking the ETS DNA-binding domain[222]. However, the mechanism of this transactivational repression by the ETV4 subfamily proteins still needs to be elucidated. Of note, ETV4 family members do not contain a PNT domain.

Further ETS transcription factors show cooperation with other transcription factors or directly synergize with general transcription factors[212,222,238,239].
Especially the common feature that ETV4 binding sites and AP-1 transcription factor binding sites are situated in close vicinity in several promoters leads to cooperativity in DNA-binding and transactivation[238,240]

In addition, the genomic structure of the ETV4 subfamily genes is very similar, suggesting that they originate from a common ancestor but diverged throughout evolution and are to-date located on different chromosomes in humans (fig. 9)[241]. The genes were mapped for ETV1 to chromosome 7 (7q22)[242], ETV4 is found on chromosome 17 (17q12)[243] and ETV5 is located on chromosome 3 (3q27)[244].

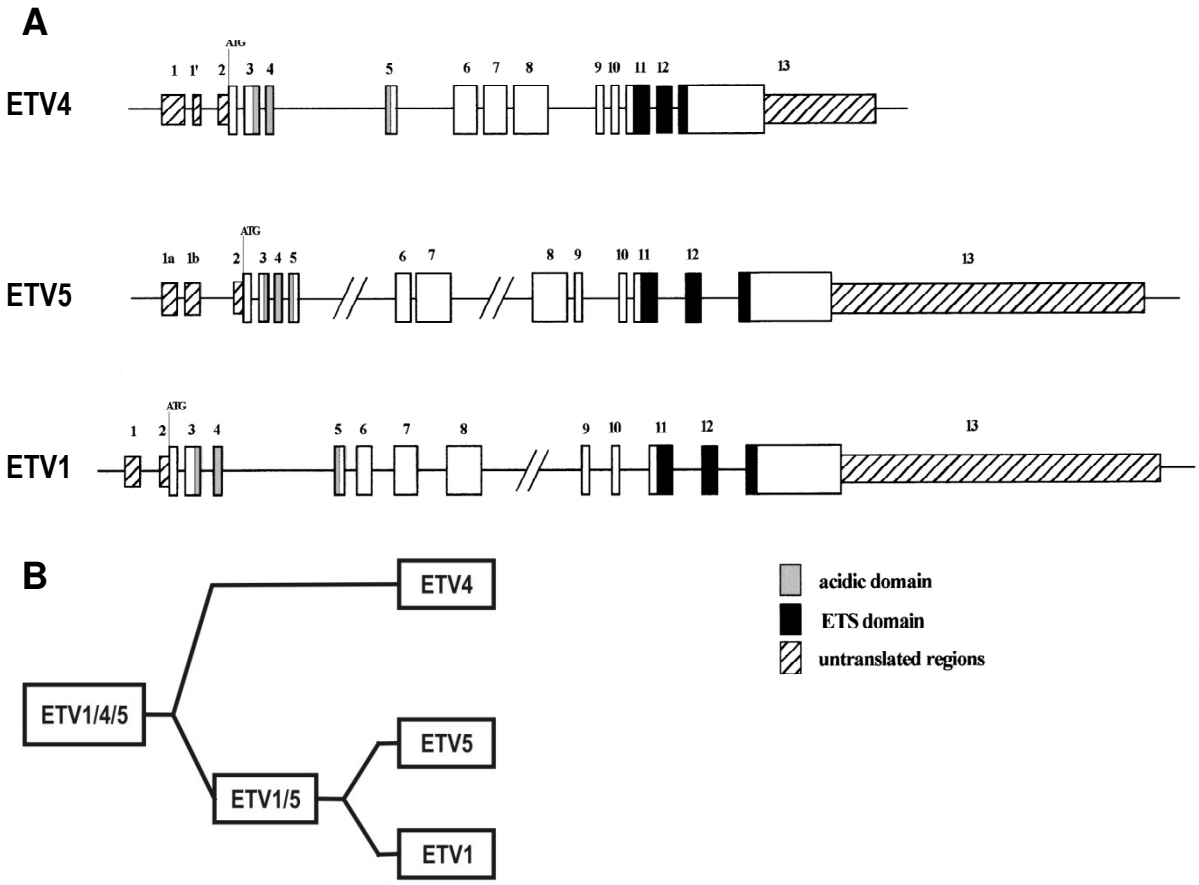

Figure 9. The genomic structure and evolution of the ETV4 subfamily genes.

(A) All three ETV4 subfamily genes have equivalently-sized exons encoding similar regions of their respective proteins. Introns are represented by a horizontal line, and exons are illustrated by rectangles. The cross-hatched areas represent non-coding, untranslated sequences embodied in the mRNAs of each gene. The sequence (ATG) encoding the translation initiation codon is present in exon 2 of each gene. The ETS domain is encoded by exons 11, 12, and 13. The acidic transactivation domain is encoded by exons 3, 4, and 5. The divergence in structure amongst these three genes occurs primarily in the untranslated regions as well as in the size of several introns (adapted from T Shepherd and AD Hassell[245]). **(B)** Phylogenetic tree of the ETV4 subfamily genes. The genes of the ETV4 group probably appear after two duplications of a common ancestor (adapted from Y de Launoit *et al.*[232]).

1.3.1.2 Discovery and characterization of ETV4

ETV4 was originally discovered as polyomavirus enhancer binding activity[246]. Four years later JH Xin and colleagues successfully cloned cDNAs that encode ETV4 from a mouse FM3A cell cDNA library through its ability to bind to the polyomavirus

enhancer element 5'-AGGAAG-3'[247]. ETV4 showed to be able to transactivate a minimal promoter containing several ETV4 binding sites[235,248]. ETV4 binding sites have been found in promoter regions of several genes of growth factors and growth factor receptors (Her2/neu) as well as proteases and their inhibitors (MMP-7, TIMP-1)[249–251].

1.3.1.3 Regulation of the ETV4 subfamily members

The regulation of the ETV4 subfamily members is suggested - like many transcription factors - to be governed by posttranslational modification, such as phosphorylation. ETV4 is mainly phosphorylated on serine residues by the MAPK pathway including RAS, RAF-1, MEK, ERK-1, and ERK-2[252,253]. The ETV4 subgroup members become phosphorylated on specific serine and threonine residues that usually results in an increase of transcriptional activity[252,237,254,255]. Additionally, ETV1 and ETV5 were shown not only to be phosphorylated by MAPK, but also by protein kinase A (PKA)[237,256–258]. The phosphorylation by PKA was shown to increase the transcriptional activity of ETV5[237,256].

Posttranslational modification at lysines has been reported to effect the regulation of transcription factors. It has been demonstrated that activation of p300 via the Ras/MAPK pathway can lead to ETV1 lysine acetylation that eventually results in an enhanced transcriptional capacity[259,260].

Another lysine modification, namely SUMOylation, was reported for the ETV4 subfamily[261–266]. SUMOlyation of ETV5 results in inhibition of transactivation, whereas SUMOylation of ETV4 is required for maximal activation of target gene promoters, including MMP-1 and COX-2, through the synergistic activation with the coactivator CBP. Furthermore, SUMOylation of ETV4 is required for ubiquitination of ETV4 and promotes its degradation, suggesting that SUMO-mediated recycling of ETV4 plays a role in ETV4-mediated promoter activation[264].

The ETV4 subfamily of transcription factors is also known to be ubiquitinated and finally degraded via the ubiquitin-proteasome pathway[267–270]. Recently, in particular

ETV1, but also ETV4 and ETV5, has been shown to be degraded by the ubiquitin E3 ligase COP1[269,270]. This degradation process seems to have an important influence on the tumor suppressing function of COP1 through the inhibition of ETV1 protein accumulation in prostate adenocarcinomas[269].

1.3.1.4 ETV4 subfamily tissue distribution

ETV5 is the most ubiquitously expressed ETV4 subfamily member. ETV5 mRNA was shown to be highly expressed in human and murine brain and placenta and, to a lesser extent, in lung, pancreas, and heart. Recently it was shown that ETV5 is expressed exclusively within Sertoli cells in the testis and is required for spermatogonial stem cell self-renewal[271]. Moreover, almost all human cell lines tested express ETV5 at varying levels[229].

In contrast, ETV1 and ETV4 show a more restricted mRNA expression pattern in human and mouse[229,248,272]. In human tissues, ETV1 mRNA is highly expressed in brain, testis, lung and heart, moderately in spleen, small intestine, pancreas and colon, weakly in liver, prostate and thymus, very weakly in skeletal muscle, kidney and ovary and not in placenta and peripheral blood leukocytes. Human ETV1 protein was found in the nucleus using immunocytochemistry. Analysis of human solid or hematopoietic tumor cell lines showed that most of them did not express ETV1 detectably[228].

The expression of ETV4 is mainly located in the adult mouse brain and male epididymis. Lower expression levels were detected in the mammary gland. ETV4 is expressed to various extents in fibroblastic and epithelial but not in hematopoietic cell lines[248].

All ETV4 subgroup members are expressed in developing mouse embryos. Interestingly ETV4 and ETV5 show overlapping expression patterns in early development stages, suggesting a redundancy in these phases[273,274]. Later the embryogenetic expression patterns of ETV4 and ETV5 diverge.

ETV1 shows a completely different expression pattern compared to ETV4 and ETV5 indicating a more divergent function during these stages[273].
The importance of the ETV4 subfamily to cell migration and invasion is currently discussed since expression of ETV4 and ETV5 was shown in numerous epithelial tissues that are often involved in branching morphogenesis (i.e. lung, salivary gland, mammary gland, kidney and the development of motor neurons) [231,273,275–279].

1.3.1.5 Genetic ablation of ETV4 subfamily genes

ETV1 knockout mice show postnatal lethality (3-5 weeks)[280]. ETV1 mutant mice exhibit a severe motor discoordination, but the specification of motor neurons and induction of muscle spindles occurs normally. The motor defect in ETV1 mutant mice results in a dramatic reduction in the formation of direct connections between proprioceptive afferents and motor neurons[280].

Although ETV4-deficient mice are viable, they show sexual dysfunctions. Male ETV4-knockout mice are indeed morphologically normal and spermatogenesis yields functional sperm. However the mice are unable to ejaculate[281]. Female ETV4 deficient mice do not show - as expected from the role of ETV4 in branching morphogenesis in breast and other organs - an impaired branching of the ductal tree but rather increased numbers of terminal end buds together with an increased proportion of proliferating cells in the postnatal mammary gland[282].
ETV4 mutant mice showed further that axons of specific motor neuron pools failed to branch normally within their target muscle and that the cell bodies of these motor neurons are mispositioned within the spinal cord[278].

ETV5-deficient mice have a defect in maintaining the spermatogonial stem cell self-renewal without a block in normal spermatogenic differentiation and thus develop progressive germ-cell depletion, eventually leading to a Sertoli-cell-only syndrome[271]. This indicates that ETV5 loss has other effects besides lack of spermatogonial stem cells self-renewal that impair fertility[283]. Interestingly, microarray analysis of primary Sertoli cells from ETV5-deficient mice showed alterations in secreted factors known to regulate the hematopoietic stem cell niche[271]. Further it was shown that ETV5-

deficiency causes an abnormal first wave of spermatogenesis, the production of specific Sertoli cells chemokines is impaired and defects in the formation of a blood-testis-barrier are found[284–286].
Female ETV5-deficient mice showed a complex reproductive phenotype deficiency such as ovarian tissue architecture defects and decreased ovulation that rendered the mice infertile[287].

ETV4 and ETV5 are involved in branching morphogenesis of the developing mouse kidney and lung as well as in the limb outgrowth[277,276,288]. Of notice, in the kidney development overlapping expression of ETV4 and ETV5 in ureteric bud tip cells is positively regulated by glial cell line-derived neurotrophic factor (GDNF) and the receptor tyrosine kinase Ret signaling pathway. It could be shown that ETV4 and ETV5 are jointly needed for a successful kidney development, since mice lacking both ETV4 alleles and one ETV5 allele have a high frequency of renal agenesis (absence of the kidney) or hypodysplasia (severe malformation of the kidney) owing to branching defects, whereas mice completely deficient in ETV4 and ETV5 show a complete absence of kidney development[277].

1.3.1.6 Physiological and pathophysiological functions of the ETV4 subfamily

The role of the ETV4 subfamily members in normal tissue development and in the adult

During chick and mouse development, ETV1 and other members of the ETV4 subfamily are expressed in several embryonic tissues and exhibit combinatorial expression in specific regions of various organs[273,274]. Later during development, ETV1 is expressed in cortical layer V neurons, motor neuron pools, and subsets of muscle sensory neurons in the spinal cord[289,280,290]. ETV1 is expressed in a subpopulation of inferior olive neurons in the hindbrain which send their projections to the caudal cerebellum[291].

ETV4 is only expressed in the adult brain and epididymis[248]. However, in the developing organism ETV4 is expressed in various cell types especially those that

proliferate or migrate[273]. Frequently the expression of ETV4 overlaps in those cells with the expression of the other subfamily members, in particular with ETV5 [273,274].
In the developing mouse lung ETV4 and ETV5 are initially restricted to the distal buds. ETV5 is transcribed exclusively in the epithelium, while ETV4 is expressed in both epithelium and mesenchyme[276].

The phenotype of ETV4 knockout mice suggests that the sterility of male mice is due to a neurological impairment. Guidance of motor and sensory neurons might be damaged and innervations of penile smooth muscles are disrupted resulting in erectile dysfunction in adult ETV4-deficient mice. Livet *et al.* underlined in 2002 this hypothesis further, when they showed that ETV4 expression controls peripheral signals that are required to coordinate the central position and terminal arborization of specific sets of spinal motor neurons in mouse[278]. Induction of ETV4 expression through the glial cell line-derived neurotrophic factor (GDNF) is essential in two cervical motor neuron pools to control dendrite patterning and selectivity of 1a proprioceptive afferents[292]. Similar results were obtained in chicken developmental studies, where ETV1 and ETV4 are selectively expressed in motor neuronal pools that innervate specific muscles[289].
Further, the role of ETV4 in the branching morphology of the adult mammary gland, kidney and lung development, as well as the muscle regeneration through the proliferation and differentiation of satellite cells, involved in muscle repair, is discussed[293,279,277,276].

ETV5 protein is localized in granulose cells of the adult mouse ovary[294]. In cycling female mice, ETV5 mRNA is additionally detected in the corpora lutea[295].

The role of the ETV4 subfamily members in tumorigenesis and metastasis

The overexpression of ETS genes, including members of the ETV4 subfamily, has been associated with breast cancer and prostate carcinomas. ETV4 subfamily members are expressed in human mammary epithelial cell lines and in the vast majority of human breast tumor cell lines, but also colon and prostate carcinomas and their cell lines[296,238]. The ETV4 subfamily proteins can cooperate with JUN, β-catenin and T-cell factor/ lymphoid enhancer factor-1 (TCF/LEF-1) to increase the expression of the target gene MMP-7 (also known as matrilysin)[238]. Consecutively,

MMP-7 expression, like that of other MMPs, can lead to increased metastasis and tumor invasion.
This was remarkably shown when the human nonmetastatic breast cancer cell line MCF-7 could be rendered invasive by the transfection of ETV4[297]. This was probably due to the ETV4-mediated induction of several matrix metalloproteinases (MMP-1, MMP-3, MMP-9) that are important for the extracellular matrix breakdown[298,299,251].

ETV4 is overexpressed in 76% of human tumor samples assessed through *in situ* hybridization[300]. In turn, especially ETV4 and its role in tumorigenesis and metastasis formation is discussed. ETV4 is known to be overexpressed in breast, ovarian and prostate cancer, oral squamous cell carcinoma, esophageal adenocarcinoma, colorectal cancer, non-small-cell lung cancer as well as in Ewing's sarcoma[300–310].
Human epidermal growth factor receptor 2 (HER2/neu), also known as ErbB-2, is a receptor tyrosine kinase that is often linked to invasiveness and metastatic inclination in breast tumors which expression results in a poor prognosis for patients[311]. HER2/neu was shown to cause in a breast cancer induced mouse model elevated levels of ETV4 mRNA in mammary adenocarcinomas and originating lung metastases[310].
In human breast cancer patients ETV4 has been demonstrated to be overexpressed compared to healthy mammary epithelial cells, as shown by *in situ* hybridization[300]. Furthermore, it was found that in more than 90% of HER2/neu expressing tumors also ETV4 is overexpressed[300]. A positive feedback loop is suggested since ETV4 is able to activate its own transcription through its promoter and ETV4 is needed to efficiently mediate HER2/neu transcription via an ETV4 binding site[249,250,300,312,313]. This loop would keep ETV4 and HER2/neu expression elevated, maintaining the cancerous phenotype.
In contrast, ETV4 has been demonstrated to repress the HER2 promoter in cell culture and to reduce the tumorigenic potential of HER2/neu in cell culture and immunodeficient mice[314]. This conflicts with most published data where ETV4 was shown to be a transcriptional activator and has been demonstrate to be overexpressed in HER2/neu-positive breast cancer in human and mouse[300,315,248,238,310].

In line with those observations, RNAi-mediated transient knockdown of ETV4 reduced invasiveness of squamous cell carcinoma-derived cell lines through the decreased expression of MMP-1, MMP-3 and MMP-9[303].

ETV5 is overexpressed in ovarian cancer and was confirmed to be important for cell adhesion regulation and thus enhancing cancer cell survival through the induced expression of adhesion molecules[316]. Interestingly, mouse mammary cancer cells tumorigenesis could be reduced upon siRNA- or shRNA-mediated inhibition of ETV4 and ETV5[317].

Chromosomal rearrangements that result in high level expression of ETS gene family members are common events in human prostate cancer[318]. Fusion proteins involved in human prostate cancer often implicate ERG, ETV1, ETV4 and ETV5 that are highly expressed upon gene combination. The fusion partners are diverse, but are mostly androgen-activated genes[318–320]. ETS gene translocations represent an early event in prostate cancer but seem to be insufficient on their own to induce cancer formation[318,320].

In fact, the two ETS genes ERG and ETV1 are highly expressed in nearly all prostate tumors. ETV1, either truncated or full length, is expressed in ≈10% whereas gene fusions involving ETV4 and ETV5 have been reported to occur in 6% and <1% of prostate cancers, respectively. The idea is widely supported that ETS overexpression drives early stages of prostate cancer development[269,318,319].

The Ewing's sarcoma and related cancer, an early documented translocation process in bone or soft tissue cancer entails a translocation between the EWS and an ETS gene, such as ETV1 and ETV4[242,306,321–327]. The translocations result in the formation of chimeric transcription factors: the N-terminus is often formed from the RNA-binding protein EWS that initiates transcription; the C-terminus is made from an ETS transcription factor supplying the ETS DNA-binding domain. Those mutant fusion proteins have been demonstrated to have a higher transcriptional activity than the wildtype proteins and might contribute to oncogenic development by altering the expression of target genes.

1.4 Aims of the thesis

PHD2 is the most ubiquitous PHD family member and supposed to be the main cellular oxygen sensor in the body. So far, besides the activation by HIF-1 little is known about the regulation of the *PHD2* gene in hypoxia[328].

1.4.1 Working hypotheses

- Not only PHD2 enzyme activity but also expression levels are of importance for regulation of the hypoxic response. This regulation is predominantly linked to the conserved sequence and activity of the promoter region.
- Being central to cellular oxygen homeostasis, it is conceivable that this feedback loop is regulated by transcription factors in addition to HIF.
- The expression of such transcription factors might influence PHD2 expression levels and thus the HIF-PHD feedback loop.
- Further understanding of the regulation of the cellular oxygen sensors might be of importance in therapeutic approaches in hypoxia-related conditions.

1.4.2 Specific aims

These hypotheses lead to the conceptual design and experimental confirmation of the following specific aims of this thesis:

- to characterize the human *PHD2* promoter in detail by serial truncations of the *PHD2* promoter region thereby assessing the minimal and conserved *PHD2* promoter region
- to set up a high throughput synthetic transcription factor screening for the identification of HIF-dependent and -independent co-activators involved into *PHD2* gene transcription
- to characterize these transcription factors and their implication in the overall transcriptional response to hypoxia

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2 Basal and inducible regulation of the oxygen sensing proly-4-hydroxylase domain 2 (PHD2) gene product

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ABSTRACT

Oxygen availability as well as insufficient oxygen supply regulate physiological adaptive processes and influence diseases such as cancer. The three human prolyl hydroxylase domain (PHD) proteins 1 to 3 are known as cellular oxygen sensors, acting via the degradation of hypoxia-inducible factor (HIF) α-subunits. Amongst the PHDs, PHD2 is most widely expressed whereas PHD1 and 3 show a more organ-restricted expression pattern. The human *PHD2* gene is driven by a promoter region located in a CpG island immediately upstream of the translational start site.

We report that the transcriptional start site of the human *PHD2* gene is located more than 318 nucleotides upstream of the translational start site. Hypoxic PHD2 expression was shown on an endogenous level to be mediated through HIF-1. Additionally we identified and cloned 95 and 55 nucleotide long *PHD2* promoter regions as highly conserved in several organisms. These regions encompassed a single HIF-binding site (HBS) and demonstrated strong hypoxia-inducibility. In this region, various putative transcription factor binding sites have been predicted. The ubiquitous transcription factor specificity protein 1 (SP1) was involved neither in normoxic nor hypoxia-induced expression of the *PHD2* gene despite the prediction of numerous SP1-consensus motifs suggested so. Total abrogation of the hypoxic response was observed, when motifs located 5' or 3' to the HBS were mutated, but the binding of the HIF-1 complex was not affected. This suggests that the three-dimensional structure of the HBS is determining HIF binding or that other transcription factors might contribute to hypoxic gene activation of the *PHD2* promoter, e.g. by stabilizing the HIF-DNA interaction or by serving as co-activators.

2.1 Introduction

A disparity between oxygen transport and oxygen consumption inevitably leads to insufficient oxygenation and metabolic starvation of the affected tissue. Hypoxia-inducible factors (HIFs) mediate the adaptation of hypoxic cells to these environmental conditions[1]. Three human prolyl-4-hydroxylase domain proteins, PHD1-3, are known as cellular oxygen sensors acting via the degradation of HIF (hypoxia-inducible factor) α-subunits[2]. Interestingly, two of the three *PHD* genes are inducible by HIFs, establishing a negative feedback loop[3–8]. We recently demonstrated that PHDs are capable of functionally regulating HIFα-subunits even under hypoxic conditions[8]. Thus, not only oxygen availability but also PHD abundance and activity regulate the HIF system[8–12].

Amongst the PHDs, PHD2 is most widely expressed, whereas PHD1 and PHD3 show a more organ restricted expression pattern[13,14]. The human *PHD2* gene is driven by a promoter region located in a CpG island immediately upstream of the translational start site[3]. Whereas the HIF effector system is well-studied, relatively little is known so far about the regulation of the PHD oxygen-sensing system. Regarding the numerous attempts to generate drugs affecting the PHD/HIF system, a better understanding of the mechanisms regulating PHD function and abundance will have important implications for basic science as well as for clinical application[15–22]. It is suggested that other important binding activities might contribute to hypoxic gene activation, e.g. by stabilizing the HIF-DNA interaction or recruitment of essential coactivators. Indeed, all three PHD genes have been shown to respond to certain upstream regulatory pathways involving estrogen stimulation, p53 activation, the proto-oncogene c-Jun, TGF-β – and hypoxia itself[5–8].

The *PHD2* promoter region defined by Metzen *et al.* was recently shown to be active under basal and hypoxia-induced conditions and successfully used in a novel synthetic screening approach that involves site-specific transcription factor interplay[3,23]. The *PHD2* promoter region has not yet been fully characterized as a result of an inaccurate description of the transcription start site. In an attempt to conclusively annotate the transcription start site of the *PHD2* gene, we referred to the 5'-rapid amplification of complementary DNA ends (5'-RACE) technique, but failed to map the 5'-end of the promoter region (data not shown). We were not

successful to establish a reliable amplification reaction in this GC-rich region. The difficulty of this endeavor is underlined by a scarce literature on the PHD2 transcriptional start site. The assembly of expression sequence tags (ESTs) equally was not elusive for the precise mapping of the transcription start site. Most of the ESTs were located in the very 3'-end of the gene which seems to be functionally irrelevant. Two transcription start sites based on the ESTs assembly were predicted in front of the first translated exon. One of them seems to rather be endorsed to an adjacent gene. The other predicted transcription start site presented a higher confidence score, but was not yet functionally proven.

In this work, we aimed to study the *PHD2* promoter architecture. Therefore, we narrowed down the transcriptional start site of the human *PHD2* gene and found that motifs located 5' or 3' adjacent to the HIF-binding site (HBS) are highly important for hypoxia-induced PHD2 expression. This suggests a sterical involvement of the HBS-adjacent regions in binding of HIF or other transcription factors in hypoxic gene activation of PHD2 expression. The gained information about the regulation of PHD2 expression helped to understand the basics of the HIF-PHD2/3 feedback regulation and in a broader perspective the transcriptional regulation of hypoxic genes.

2.2 Results

2.2.1 Narrowing of the transcriptional start site location in the *PHD2* promoter

Previously we have reported on a functional, single HBS located in the 5'-regulatory region of the human *PHD2* gene[3,23]. Alignment of the *PHD2* promoter region of various vertebrates revealed that the HBS surrounding region is conserved to approximately 66% (fig. 1A). Although, the predicted *PHD2* promoter was confirmed to be functional, the transcriptional start site and with this the precise location of the *PHD2* promoter remains unknown. In an attempt to map this important part of the promoter, 3'-truncated luciferase reporter gene constructs were generated. A region spanning from -1070/+3 (relative to the translation start site) showed maximal basal activity when transfected into U2OS (human osteosarcoma) and HeLa (human cervix carcinoma) cells[23]. The 3' truncation, named P2P (-1070/-318), was cloned into a luciferase reporter gene vector, basal as well as hypoxic activation of the reporter were analyzed and compared to the P2P (-1070/+3) construct in U2OS and HeLa cells using a dual luciferase readout assay (fig. 1B). These reporter gene constructs harbour a translation start site for the reporter gene (luciferase), while transcriptional initiation by RNA polymerase II is mediated by the *PHD2* promoter construct. If the transcription start site had been deleted, luciferase mRNA, protein and eventually reporter activity levels would be blunted to background levels. With the deletion of 318 nucleotides upstream of the translational start site, luciferase expression still takes place, suggesting that the transcriptional start site is located further upstream of the eliminated 318 nucleotides (fig. 1C and D). Additionally, hypoxic induction was strictly dependent on a functional HBS, since point mutations of the binding site completely abolished the response to hypoxia in either cell line (fig. 1C and D).

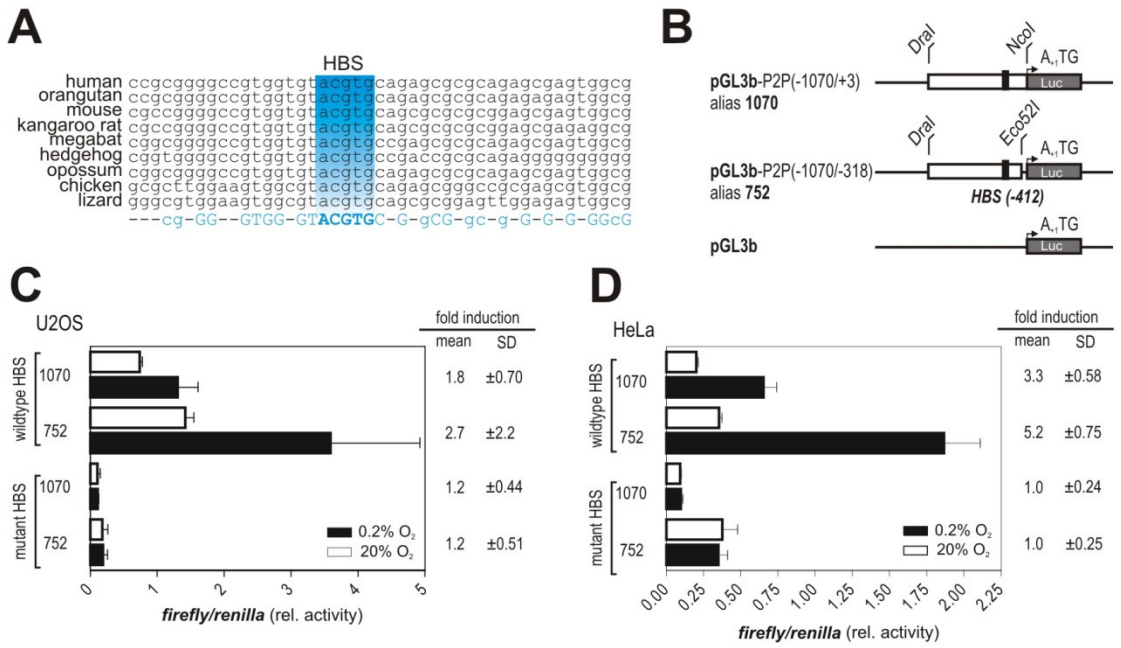

Figure 1. The transcription start site of the human *PHD2* gene is located more than 318 nucleotides upstream of the translational start site.

(A) Alignment of the *PHD2* promoter region in various vertebrates revealed a conservation of the HBS surrounding region. **(B)** Illustration of the *PHD2* promoter (P2P) 5'- and 3'- truncation and its cloning strategy. The functional HIF-binding site (HBS) is indicated by a black box. **(C)** Constructs as depicted in *B*. were transiently transfected into human osteosarcoma (U2OS) or the cervix carcinoma cell line HeLa (right panel), respectively. After 24 h of transfection, cells were incubated for further 24 h under 20% O_2 or 0.2% O_2. Bars represent means ± S.E.M. of three independent experiments performed in quadruplicates. The HBS was mutated from CGTGC to ATAAT. Induction factors were calculated based on hypoxic reporter gene activation versus basal reporter activity at 20% O_2. Data are given as mean ± S.D.

2.2.2 The *PHD2* promoter is regulated by HIF-1

The further characterization involved to typify the HIF-binding properties of the *PHD2* promoter. The core consensus motif 5'-RCGTG-3' was defined by the analysis of about 70 HIF-target genes[2]. Such a HBS motif is also found in the *PHD2* promoter and has been shown to be functional[3]. However, this motif does not allow discriminating if hypoxia-induced target gene expression is driven by HIF-1 or HIF-2[24]. Aprelikova *et al.* already gave indications by overexpression and silencing experiments that HIF-1, but not HIF-2 binding causes the hypoxia-induced PHD2 expression[25]. We wanted to confirm this finding in two experimental settings.

First, in *Hif1a*-deficient mouse embryonic fibroblasts (MEFs) (fig. 2A inlay) HIF-α expression was rescued by transfecting vectors overexpressing either HIF-1α or HIF-2α or in control settings β-galactosidase. The *PHD2* promoter construct pGL3 P2P (-607/+3) - previously shown to have highest hypoxic induction while maintaining basal transcription levels[23] - was additionally co-transfected. After treatment for 24 hours under normoxic or hypoxic conditions, luciferase reporter activity was analyzed. Forced expression of both HIF-1α and HIF-2α could induce normoxic PHD2 expression concluding that PHD2 expression could be rescued. However, only overexpressed HIF-1α, but not HIF-2α, amplified the hypoxic PHD2 expression. In case of a mutated HBS, none of the HIF-α subunits could activate the promoter region (fig. 2A).

To confirm the obtained result in an endogenous setting we used MCF7 cells in an second approach known to express functional HIF-1α and HIF-2α subunits (fig. 2B) for chromatin immunoprecipitation (ChIP) experiments using either anti-HIF-1α or anti-HIF-2α antibodies. Oxygen-dependent enrichment of the HRE-containing *PHD2* promoter region was mainly restricted to the HIF-1α precipitations (fig. 2C), providing further evidence for the preferential recruitment of HIF-1 over HIF-2 to the endogenous *PHD2* locus.

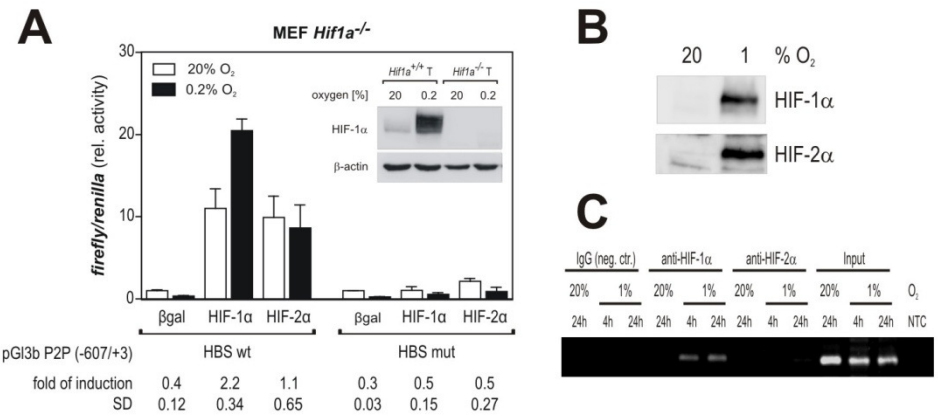

Figure 2. The *PHD2* promoter is regulated by HIF-1.

(A) The HBS wt or mutated form of the truncated *PHD2* promoter P2P(-607/+3) was transiently transfected into *Hif1a* - deficient mouse embryonic fibroblasts cells (MEF *HIF1a -/-*) along with either HIF-1α or HIF-2α expression vectors. The β-galactosidase (β-gal) control expression vector was cotransfected. After 24 h of transfection, cells were incubated under 20% or 0.2% O_2 for another 24 h. Bars represent means ± S.E.M. for 3 independent experiments. **(Inlay)** Rescue of HIF-1α was confirmed by Western blotting. β-actin protein levels were used as loading control. **(B)** Analysis of HIF-1α and HIF-2α expression by Western blotting of MCF7 exposed to 24 h to normoxia or 0.2% O_2 hypoxic conditions. **(C)** Chromatin immunoprecipitation (ChIP) of normoxic or hypoxic MCF7 cells using antibodies directed against HIF-1α or HIF-2α, control serum (IgG) or 1:50 diluted input samples. The amount of co-precipitated chromatin derived from the human *PHD2* promoter region containing the HBS was determined by PCR followed by agarose gel electrophoresis.

2.2.3 Conserved HBS-encompassing *PHD2* promoter element and its effects on PHD2 expression

Little is known about target gene regulation by HIF in cooperation with adjacent transcription factors. As the HBS is directly located within the promoter region, it is surrounded by multiple putative binding motifs for other transcriptional regulators. Computational analysis of murine and human *PHD2* promoter regions only show few sequence similarities, but interestingly a stretch of 95 nucleotides (P2P(-465/-370) wt) encompassing a single HBS could be identified as highly conserved.

The 5'-region of the conserved promoter element shows a strong accumulation of putative SP1-binding sites. In the 3'-end putative transcription factor binding sites were predicted *in silico* linked with two cell cycle-dependent factors (e.g. cell growth defect factor 1 (CDF-1); cyclin D binding myb-like transcription factor 1 (DMP1)).

The conserved region of the *PHD2* promoter, as well as a 5' truncated 55 base pair long version - where most of the predicted SP1 binding sites were eliminated -named P2P(-425/-370) wt (depicted in fig. 3A) were cloned into luciferase reporter gene vectors. After transient transfection into U2OS and HeLa cells and incubation under 20% O_2 or 0.2% O_2 for 24 hours, dual-luciferase activity was determined. Remarkably, those short regions were sufficient to confer highest hypoxic luciferase expression without any heterologous promoter in U2OS and HeLa cells (fig. 3B).

In order to disrupt potential transcription factor binding sites, a selected subset of predicted binding sites was mutated by hexanucleotide mutations (mut) (5'-AAAAAA-3') within the conserved 55 nucleotide *PHD2* promoter region and inserted into a dual luciferase readout system **(**fig. 3C). Two of these mutated constructs, namely those motifs located 5' or 3' adjacent to the HBS (mut A and B) completely lost hypoxic inducibility, even though the functional HBS, previously shown to be sufficient for HIF-1 binding, was preserved[26]. Mutation C (predicted binding site for SP1) and mutation D (predicted binding site for CDF-1) strongly reduced hypoxic reporter gene activation. Mutation E (predicted binding site for DMP1) shows, if at all, only a limited effect on PHD2 expression (fig 3D). Similar results were obtained when the experiment was repeated in HeLa cells (fig 3D). This suggests that other transcription factors might contribute to hypoxic gene activation, e.g. by stabilizing the HIF-DNA interaction or by serving as coactivators.

However, neither Sp1-deficient MEFs nor basal or hypoxic PHD2 expression was affected when Sp1 expression was rescued. Mutation of the HBS completely abrogated hypoxic induction independently of SP1 (fig. 3E).

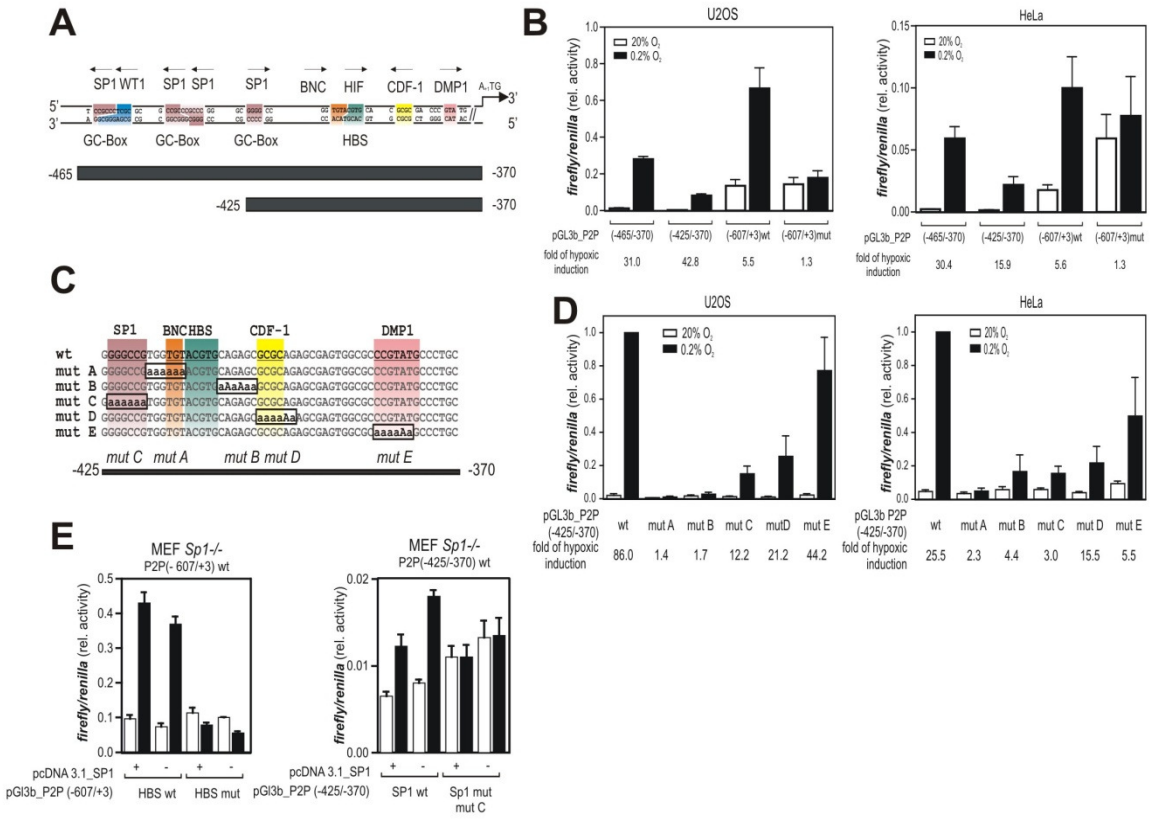

Figure 3. Determination of regulatory elements in close vicinity to the HBS

(A) Illustration of the conserved 95 bp and 55 bp long *PHD2* promoter elements present in the constructs P2P(-465/-370)wt and P2P(-425/-370)wt, and its predicted transcription factor binding sites with consensus sequences: BNC – basonuclin 1; CDF-1 - cell growth defect factor 1; DMP1 - cyclin D binding myb-like transcription factor 1; HBS – HIF binding site; SP1 – specificity protein 1; WT-1 – Wilms tumor 1 **(B)** Luciferase reporter gene assays of P2P(-465/-370)wt, P2P(-425/-370)wt, P2P (-607/+3) wt and mut. The constructs were transiently transfected into human U2OS and HeLa cells and 24 h after transfection, cells were incubated under 20% O_2 or 0.2% O_2 for another 24 h. **(C)** Scheme of hexanucleotide mutations (5'-aaaaaa-3') and affected transcription factor binding sites within the *PHD2* promoter element. **(D)** Luciferase reporter gene assays of P2P(-425/-370)wt and its binding site mutants A-E. Constructs as depicted in *C.* were transiently transfected as described in *B*. **(E)** Luciferase reporter gene assays of the wildtype or mutated form. The wt or mutated *PHD2* gene regulatory region of P2P(-607/+3) (left graph) or P2P(-425/-370) (right graph) were transiently transfected into *Sp1*- deficient mouse embryonic fibroblasts (MEF *Sp1* -/-) along with either SP1 (+) or β-gal (-) expression vectors. All bars represent means ± S.E.M. of 3 independent experiments performed in quadruplicates.

2.2.4 HIF binding to the *PHD2* promoter stretch

As the efficient abrogation of hypoxic reporter activation by mutation of the surrounding sequences might be due to impaired binding of HIF-1, we performed electromobility shift assay (EMSA) using nuclear extracts from HepG2 cells cultured at either 20% or 0.2% O_2. In line with the dual-luciferase data EMSA revealed that mutations close to the HIF consensus sequence 5'-RCGTG-3' do not impair the binding of HIF-1 (fig. 4A). While mutation of the HBS completely disrupted specific HIF-1 binding, oligonucleotides containing the flanking hexanucleotide mutations were still bound by HIF-1, albeit to a somewhat reduced extent. Interestingly, a hypoxia-inducible binding activity that was unaffected by mutation of the core HBS co-migrated with the HIF-1 complex (fig. 4A *(i.)*).

To obtain a higher resolution, 2-nucleotide mutations to adenosines were introduced in the GC-rich HBS-flanking regions (fig. 4B). The oligonucleotides containing the flanking dinucleotide mutations of the conserved 55 nucleotides *PHD2* promoter region were subsequently inserted into a luciferase reporter gene system. After transfection and treatment for 24 hours under normoxic or hypoxic conditions, luciferase activity was determined (fig. 4C). Interestingly, mutation 1 showed an increase in both normoxic and hypoxic levels, possibly through the mutation of a binding sequence of an inhibitory element. Mutations closer to the HBS reduced hypoxic inducibility and overall normoxic and hypoxic expression levels, although this reduction in PHD2 expression was not as pronounced as the complete abrogation through a mutation of the entire HBS. These findings suggest regions surrounding the HBS to influence PHD2 expression either for sterical reasons or through the interaction with other transcription factors binding adjacent to the HBS. The alignment of the HBS-region in the *PHD2* promoter of various vertebrates showed certain conserved nucleotides 5' and 3' of the RCGTG motif (fig. 1A). In combination with the results obtained from dinucleotide mutations that suggest the additional importance of 2-3 nucleotides around the HBS we propose an enlarged core region of 5'-GT**ACGTG**CX-3'.

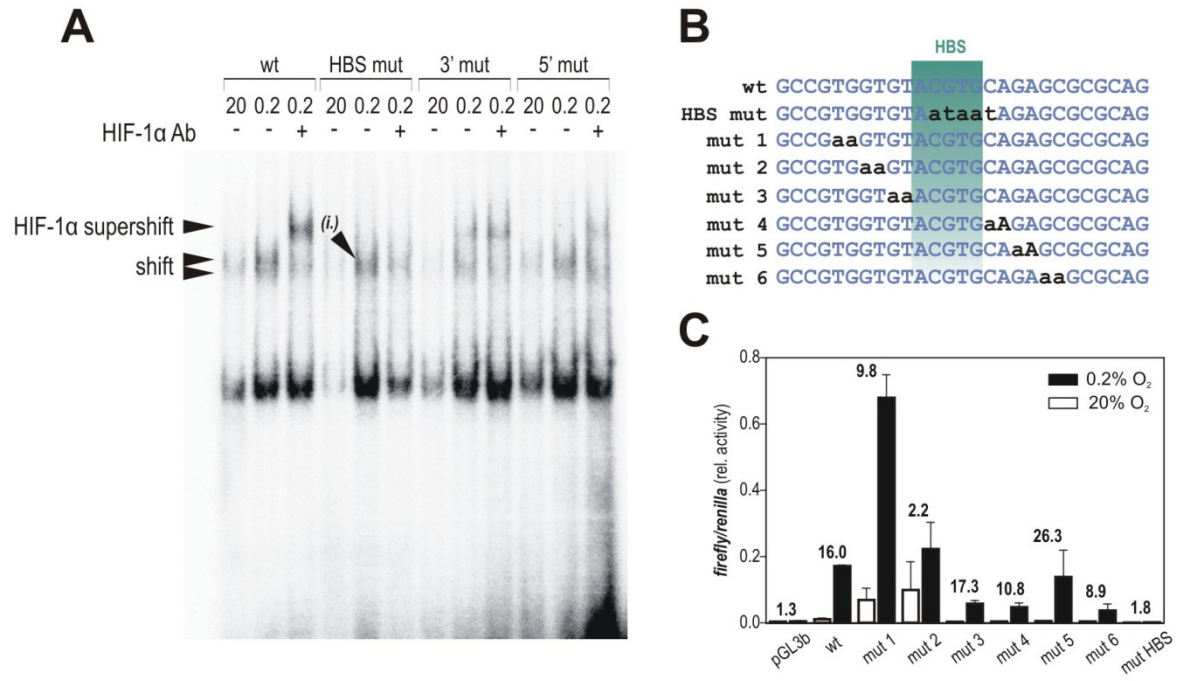

Figure 4. Analysis of the *PHD2* promoter region in close vicinity to the HBS

(A) EMSA of nuclear extracts from HepG2 cells cultured at 20 or 0.2% O_2 for 6 h. HIF binding to *PHD2* promoter oligonucleotides (27 bp) (depicted in fig. 3C) encompassing the HBS surrounding sequence with or without indicated mutations. Specificity of the HIF-1 signal was demonstrated by supershift analysis with an anti-HIF-1α antibody.***(i.)*** The arrowhead points to a band of a hypoxia-inducible binding activity that is unaffected by mutation of the core HBS. **(B)** Scheme of dinucleotide mutations (5'-aa-3') (bold) around the HBS within the 55 bp long GC-rich *PHD2* promoter element P2P(-425/-370)wt. **(C)** The 55 nucleotides encompassing regulatory element of the human *PHD2* promoter was cloned into the promoterless pGL3basic luciferase reporter gene. Constructs as depicted in *B.* were transiently transfected into U2OS cells and treated as described in *fig. 3B*. Data are given as mean ± S.E.M.

2.3 Discussion

The three human PHD proteins 1 to 3 are known as cellular oxygen sensors acting via the degradation of HIF-α subunits[1,9,27–31]. Amongst the PHDs, PHD2 is most widely expressed, whereas PHD1 and PHD3 show a more organ restricted expression pattern. However, little is known about the regulation of the *PHD2* gene itself. Therefore we performed promoter analysis to study the transcriptional regulation of the *PHD2* gene and to gain a better understanding of the underlying mechanisms in respect to hypoxia[9,32]. Interestingly, the *PHD2* and *PHD3* genes are inducible by HIFs themselves, establishing a negative feedback loop[3–7,33]. Thus, not only oxygen availability, but also PHD abundance regulates the hypoxic response[33]. We suggest that other transcription factors might contribute to hypoxic gene activation, e.g. by stabilizing the HIF-DNA interaction or by serving as co-activators. Therefore, we studied the promoter architecture and function in order to find further regulatory mechanisms of *PHD2* gene expression.

2.3.1 The transcription start site of the *PHD2* gene

The *PHD2* gene has been mapped to chromosome 1 and consists of 5 exons[34]. In 2005, Metzen *et al.* suggested through the identification of a CpG-island - a common genomic region for harboring a promoter - and various other prediction methods two different promoter sites upstream of the *PHD2* coding sequence[3]. Transcription factor binding sites prediction revealed plenty of putative binding sites in the human and murine *PHD2* gene hinting to a transcriptionally active genomic region. However, the promoter element predicted further upstream (approx. 3.5 kb 5' of the translation start site) seemed to be transcriptionally silent in adult tissue and might be of relevance in early phases of embryonic development. This hypothesis, though, lacks experimental evidence to date. Thus, *PHD2* gene expression seems to be solely driven by the functionally active promoter that is located immediately 5' of the translation start site and contains a functional HBS (-412 bp to the translation start site)[3,23]. Although, EST assembly was performed the identification of the transcription start site was not successful[3]. The EST found most upstream in the UCSC genome

browser is located at the 3'-end of the first translated exon and misses the mRNA sequence of almost the complete first exon. The predicted transcription start sites that deviated from the EST sequence alignments were dispersed in the gene. A disadvantage of ESTs and EST-based transcription start site prediction is that they are derived from single sequencing reactions and might include erroneous runs. This misinformation lowers the reliability to obtain a correct sequence and might cause problems in assembling the accurate sequence. Some of the predicted transcription start sites for the *PHD2* gene were settled in the very 3'-end which seems to be functionally questionable. Two other transcription start sites were predicted in front of the first exon. One of them has a low confidence score (21.0) and is located outside of the CpG-island. This transcription start site seems rather to be attributed to the adjacent gene (*Disc1*, encoded on the opposite strand and running in the opposite direction). The other predicted transcription start site with a higher confidence score (63.0) appears to be more potent. Although this sequence was absent in the *PHD2* promoter construct P2P (-1070/-318) the reporter was still transcriptionally active. This could mean that either the approach of deleting parts of the *PHD2* promoter in a non-endogenous context is not appropriate or there is another, not yet predicted transcription start site present in the *PHD2* promoter. Eventually, the transcription start can only be determined by experimental analysis of endogenous mRNA such as the establishment of 5'-RACE that did not succeed in our hands.

2.3.2 The prevalence of HIF-1 for the hypoxia induced induction of PHD2

The HIF-1α and HIF-2α subunits are structurally similar in their DNA binding and dimerization domains. However, they differ in their transactivation domains and have been shown to have assorted target genes. It was demonstrated that HIF-1α and HIF-2α regulate distinct target genes. HIF-1 has been reported to be primarily involved in the regulation of gene products of the glycolytic pathway, whereas HIF-2 plays a role in stem cell pluripotency[35,36]. Since a binding preference of a HIF isoform is not deducible from the HBS motif alone the further characterization of the *PHD2* promoter included to analyze the prevalence of HIF-1 or HIF-2 trans-activation[24]. In a previous report the preference of HIF-1 for the *PHD2* locus was indirectly proven by silencing or overexpressing either HIF-1α or HIF-2α. Hypoxia-induced PHD2

expression was affected neither way by HIF-2α[25]. We confirmed in two experimental settings that the hypoxic *PHD2* promoter regulation is principally conducted through HIF-1α rather than HIF-2α. In a similar experiment to the above mentioned ones we rescued hypoxic *PHD2* promoter activity through forced HIF-1α expression in MEF *Hif1a*$^{-/-}$. However, the HIF-1/2α balance of a cell can be shifted by silencing or overexpressing one of the isoforms resulting in a potential compensation process[37]. Therefore, we aimed in a second experiment to demonstrate the dominance of HIF-1 in hypoxic PHD2 expression on endogenous levels. We could show that endogenous HIF-1α is found on the *PHD2* locus in hypoxia. However, MCF7 cells were reported to express a HIF-2α isoform having little transcriptional activity[38]. It was additionally reported that HIF-1α is decreasing in prolonged hypoxia while HIF-2α protein is increased[37]. The possibility is left that the preference for a particular HIF isoform to bind to the *PHD2* promoter shifts in long-term hypoxia. Still, the preferred binding of HIF-1 at the *PHD2* locus is in line with the observation of Schödel *et al.* who provided evidence that the majority of HIF-1 binding sites is located close to the promoter site, whereas HIF-2 binding sites are more often located in large distance to the influenced gene region[24]. As a matter of fact, the functional HBS is situated 412 bp upstream of the translation start site, within the promoter.

2.3.3 The conservation of PHD2 HBS surrounding region

As mentioned before, we found a conservation of the HBS and its surrounding sequence in human, mice and other vertebrates. We observed that the 95 bp and 55 bp *PHD2* promoter elements were sufficient to confer hypoxic inducibility, including in a murine tumor allograft model[39]. Interestingly, the regions located 5' or 3' adjacent to the HBS were shown to be highly important for hypoxia-induced PHD2 expression, even though the functional HBS, previously shown to be sufficient for HIF-1 binding, was preserved[26]. The suggested enlarged HBS motif corresponds with the observed nucleotide sequence preferences of a high-resolution genome-wide mapping of HBSs by ChIP[24]. In this unbiased approach an additional emphasize is put on the preference for a $^{C}/_{G}$T at the two positions before the HBS and a C after the RCGTG-motif. The same preference is found in the PHD2 HBS underlining the conservation and importance for hypoxia-induced regulation of the

oxygen sensor PHD2. Since roughly 50% of promoter sites are located in a CpG-island, those regions show an accumulation of SP1-binding motifs (5'-GGGCGG-3') simply because they are GC-rich. As well we predicted SP1-binding sites in the PHD2 region conserved amongst vertebrates that showed not to be functionally relevant and did not affect PHD2 expression either on basal or on hypoxic level.

In summary, we confirmed on an endogenous level that hypoxic PHD2 expression is mediated through HIF-1. We identified a 95 bp-long *PHD2* promoter region encompassing a single HBS as highly conserved in several organisms and demonstrate strong hypoxia-inducibility (even when the element was shortened to 55 bp). Total abrogation of the hypoxic response, but not of the HIF-1 complex binding could be shown in dual-luciferase assay and EMSA experiments, when motifs located 5' or 3' adjacent to the HBS were mutated. Our findings shed light on the cellular events and networks connected to PHD expression.

2.4 Material and methods

Plasmid constructs

The 95 base pair conserved *PHD2* promoter construct was previously published[39] and a 55 nucleotides truncated version of the regulatory element of the human *PHD2* promoter (P2P(-465/-370)wt and P2P(-425/-370)wt) was similarly generated. Both constructs were cloned by introducing synthetic oligonucleotides into the promoterless pGL3basic luciferase reporter gene backbone. Mutations were integrated by hexanucleotide (5' -aaaaaa- 3'), dinucleotide (5' -aa- 3') or HBS mutations (5'-CGTGC-3' to 5'-ataat-3'). *PHD2* promoter (P2P) constructs containing the wildtype and mutated HBS in pGL3basic luciferase vector were published before[3]. These constructs were modified employing a 5'-truncation and a 3'-start codon fusion to the luciferase open reading frame by using standard restriction-mediated cloning techniques. The *PHD2* luciferase reporter plasmid pGL3 P2P (-607/+3) contained *PHD2* promoter sequences extending from –607 to +3 (ATG start codon) and has been published previously[40]. Cloning of PHD2, PHD3, β-galactosidase, HIF-1α, HIF-2α and SP1 expression vectors (pcDNA3.1) was carried out using Gateway technology (Invitrogen, Basel, Switzerland) as described previously[10].

Protein extraction and immunoblot analysis

Cells were washed twice and scraped into ice-cold phosphate-buffered saline. Soluble cellular protein was extracted with a high salt buffer containing 0.1% Nonidet P-40 essentially as described before[41]. Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard, and 50–80 µg of protein were subjected to immunoblot analysis. The following antibodies were used: mouse anti-human HIF-1α (Transduction Laboratories BD Biosciences), rabbit anti-human HIF-2α (Novus Biologicals) and mouse anti-β-actin (Sigma) were detected with secondary polyclonal goat anti-mouse antibody coupled to horseradish peroxidase (HRP) (Pierce). Chemiluminescence detection was done using Supersignal West Dura (Pierce) and recorded with a CCD camera (Fuji, LAS 4000) followed by quantification with Quantity One software (Bio-Rad).

Cell culture and transfections

Human cervix carcinoma (HeLa), human osteosarcoma (U2OS), human shPHD2 breast cancer (MCF7), human hepatocellular carcinoma (HepG2) and mouse embryonic fibroblast (MEF) *HIF1a-/-* and *Sp1-/-* cells were grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum (FCS) and antibiotics (penicillin 50 IU/ml and streptomycin 100 µg/ml, Invitrogen) (Gibco-BRL) as described previously[12]. Hypoxic conditions were generated by incubation of cells in an InvivO2 400 hypoxic workstation (Ruskin Technologies). The oxygen concentration was maintained at 0.2-1%, with the residual gas being 94-94.8% nitrogen, and 5% carbon dioxide. Transfections were performed using polyethylenimine (Polysciences) as described previously[8].

Chromatin immunoprecipitation (ChIP)

ChIP assays from parental MCF7 cells exposed to 20% or 1% O_2 for 4 and 24 hours were performed essentially as described previously[37]. The following antibodies were used for immunoprecipitation: rabbit anti-HIF-1α (ab2185; Abcam), and rabbit polyclonal anti-HIF-2α IgG fraction (ab199; Abcam). Rabbit serum (011-000-001; Jackson ImmunoResearch) served as unspecific control. Enrichment of *PHD2* promoter chromatin was determined by PCR using the following primers: *PHD2* forward 5'-gtatgccctgcgctcctc-3', reverse 5'-gctgagagaatagggcctgtg-3'.

Dual-luciferase assay

Cells were cotransfected with 20 ng pRLSV40 Renilla luciferase reporter gene vector (Promega) and 3 µg reporter plasmid or 1.5 µg reporter and additionally 1.5 µg expression plasmid, respectively. After 24 hours of normoxic and further 24 hours of hypoxic (0.2% O_2) incubation cells were lysed in 20 µl passive lysis buffer (Promega). Luminescence was immediately analyzed with a microplate luminometer (Berthold) using the dual-luciferase reporter assay kit (Promega).

Nuclear extract preparation and EMSA (electrophoretic mobility-shift assay)

Nuclear extracts were prepared from HepG2 cells incubated in a 0.2% oxygen atmosphere for 4 hours as described earlier[42]. Oligonucleotides for gelshift assays were synthesized by MWG-Biotech (Microsynth, Switzerland). Sequences were

derived from the human *PHD2* gene, containing the putative HBS (PHD2-HBS, nt 2747, GenBank® accession no. AF229245) or a mutated HBS (PHD2-HBSmut):
wildtype (wt: 5'-GCCGTGGTGTACGTGCAGAGCGCGCAG-3'),
HBS mutant (HBS mut: 5'-GCCGTGGTGTAataatAGAGCGCGCAG-3'),
mutant A (mut A: 5'-GCCGaaaaaaACGTGCAGAGCGCGCAG-3'),
mutant B (mut B: 5'-GCCGTGGTGTACGTGaAaAaaGCGCAG-3')

The 5´-end ^{32}P-labeling, annealing and binding reactions were performed as described previously[42,3]. Samples were resolved by electrophoresis on native 5% polyacrylamide gels at room temperature. Gels were dried and analyzed by phosphoimaging (BAS 1000; Fuji, Düsseldorf, Germany). Specificity was tested by supershift experiments. For this purpose, 1 µl of undiluted monoclonal anti-HIF-1α antibody (BD Biosciences, Heidelberg, Germany) was added to the nuclear extract 60 min before the gel was run.

Alignment

Promoter region prediction and analysis of putative transcription factor binding sites in the human and murine *PHD2* genes were performed with ALGGEN Promo 3.0 (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3).
Sequence alignments were performed as described before using ClustalX[3].

Statistical analysis

If not otherwise indicated, results are presented as mean values ± standard error of the mean (S.E.M.) of at least three independent experiments. *P*-values were obtained by unpaired *t*-tests (*P<0.05, **P<0.01, ***P<0.001).

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3 Synthetic transactivation screening reveals ETV4 as broad co-activator of hypoxia-inducible factor signaling

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ABSTRACT

The human prolyl-4-hydroxylase domain (PHD) proteins 1 to 3 are known as cellular oxygen sensors, acting *via* the degradation of hypoxia-inducible factor (HIF) α-subunits. *PHD2* and *PHD3* genes are inducible by HIFs themselves, suggesting a negative feedback loop that involves PHD abundance. To identify novel regulators of the *PHD2* gene, an expression array of 704 transcription factors was screened by a method that allows to distinguish between HIF-dependent and HIF-independent promoter regulation. Amongst others, the E-twenty six (ETS) transcription factor ETV4 was found to contribute to *PHD2* gene expression particularly under hypoxic conditions. Mechanistically, complex formation between ETV4 and HIF-1/2α was observed by mammalian two-hybrid and FRET analysis. HIF-1α domain mapping, CITED2 overexpression and FIH depletion experiments provided evidence for cooperation between HIF-1α and p300/CBP in ETV4 binding. Chromatin immunoprecipitation confirmed ETV4 and HIF-1α co-recruitment to the *PHD2* promoter. Of 608 hypoxically induced transcripts found by genome-wide expression profiling, 7.7% required ETV4 for efficient hypoxic induction, suggesting a broad role of ETV4 in hypoxic gene regulation. Endogenous ETV4 highly correlated with PHD2, HIF-1/2α and several established markers of tissue hypoxia in 282 human breast cancer tissue samples, corroborating a functional interplay between the ETV4 and HIF pathways.

3.1 Introduction

Cellular adaptation to a shortage of oxygen is mainly governed by transcriptional regulation. Hypoxia-inducible factors (HIFs) are key players in the hypoxic cell and orchestrate the expression of hundreds of downstream target genes, adapting the cellular metabolism to a low oxygen environment[1]. Heterodimeric HIFs consist of a tightly O_2-regulated α-subunit (HIF-1α, HIF-2α or HIF-3α) and a constitutively expressed β-subunit (HIF-1β). At oxic conditions HIF α-subunits are continuously marked for proteasomal degradation through hydroxylation of two key prolyl-residues by prolyl-4-hydroxylase domain (PHD) oxygen sensor proteins[2]. PHD hydroxylation activity fades as a direct function of oxygen, thus reciprocally controlling the nuclear accumulation of HIFαs. Stabilized HIF-complexes bind to a *cis*-acting HIF binding site (HBS) conformed by a highly conserved core 5'-RCGTG-'3 motif present in all direct target genes[1,3,4]. In analogy to de-stabilizing proline hydroxylation, the transcriptional activity of HIF is tuned by factor inhibiting HIF (FIH) which hydroxylates a distinct asparagine residue within the HIFα carboxy terminus and consequently hinders its association with the transcriptional 300-kilodalton co-activator protein (p300) and CREB binding protein (CBP)[5–7].

Amongst the three characterized HIF prolyl-4-hydroxylases, PHD2 is widely accepted as the most crucial isoform controlling basal activity of the HIF pathway in oxic cells[8]. Underlining its dominant role, disruption of the *Egln1* locus, encoding mouse PHD2, results in prenatal lethality, while PHD1 and PHD3 knockout mice are born normally[9]. Broad-spectrum conditional deletions of all three PHDs in mice revealed a global hyperproliferative vascular phenotype uniquely when targeting PHD2, demonstrating an absolute requirement for PHD2 which is not confined to embryonic development[10]. Accordingly, PHD2 abundance is considered as a critical factor in tumor angiogenesis, though divergent roles of stromal and tumor cell derived PHD2 have been discussed[11–13]. As PHD2 protein is strikingly stable and the *de novo* translated enzyme outlasts a period of transient hypoxia by more than 48 hours, transcriptional regulation of the *PHD2* locus must be considered as the main process defining cellular levels of PHD2[14,15]. Expression of PHD2 itself is delicately

influenced by HIF transcriptional activity, forming a negative feedback loop which facilitates dynamic oxygen sensing[16–18].

In order to identify upstream regulatory pathways affecting *PHD2* gene expression in an unbiased system, we developed a screening approach that allows the identification of transcriptional interactions with DNA-bound HIF complexes and HIF-independent promoter regulation at the same time. The herein described synthetic transactivation screening led to the identification of several members of the E-twenty six (ETS) and FOS families of transcription factors as novel activators of the human *PHD2* promoter. Amongst those, ETS translocation variant 4 (ETV4; also known as E1A enhancer binding protein, E1AF; or polyoma enhancing activator 3, PEA3), was found to be a potent co-activator of HIF-1-dependent transcription.

3.2 Results

3.2.1 A single HIF DNA-binding site (HBS) is sufficient for hypoxic induction of the human *PHD2* promoter but dispensable for basal promoter activity

Previously, we have reported on a functional, single HIF-binding site located in the 5'-regulatory region of the human *PHD2* gene[16]. By using a series of 5'-truncated luciferase reporter genes (schematically depicted in **fig. 1A**), the minimal hypoxia-responsive region of the *PHD2* promoter was further mapped to an element spanning nucleotides -424 to +3 relative to the translational start site. While pGL-P2P(-424/+3) still revealed high hypoxic inducibility when transiently transfected into U2OS or HeLa cells, normoxic promoter activity of this region was largely lost when compared to the longer promoter variants, regardless of whether the HBS was wildtype or mutant **(fig. 1B)**. Elongation of the promoter by at least 183 nucleotides (construct pGL-P2P(-607/+3)) showed robust normoxic activity in both cell lines **(fig. 1B)**. Since basal induction of HBS-lacking constructs was observed between nucleotides -424 and -607 without any further effects upstream, the existence of transcriptionally active elements required for oxic expression of the *PHD2* gene in this particular region is suggested **(fig. 1B)**. Of note, the fold of hypoxic activation of pGL-P2P(-607/+3) resembled the oxygen-dependent induction factors of endogenous PHD2 mRNA and protein in both cell lines **(fig. 1C)**.

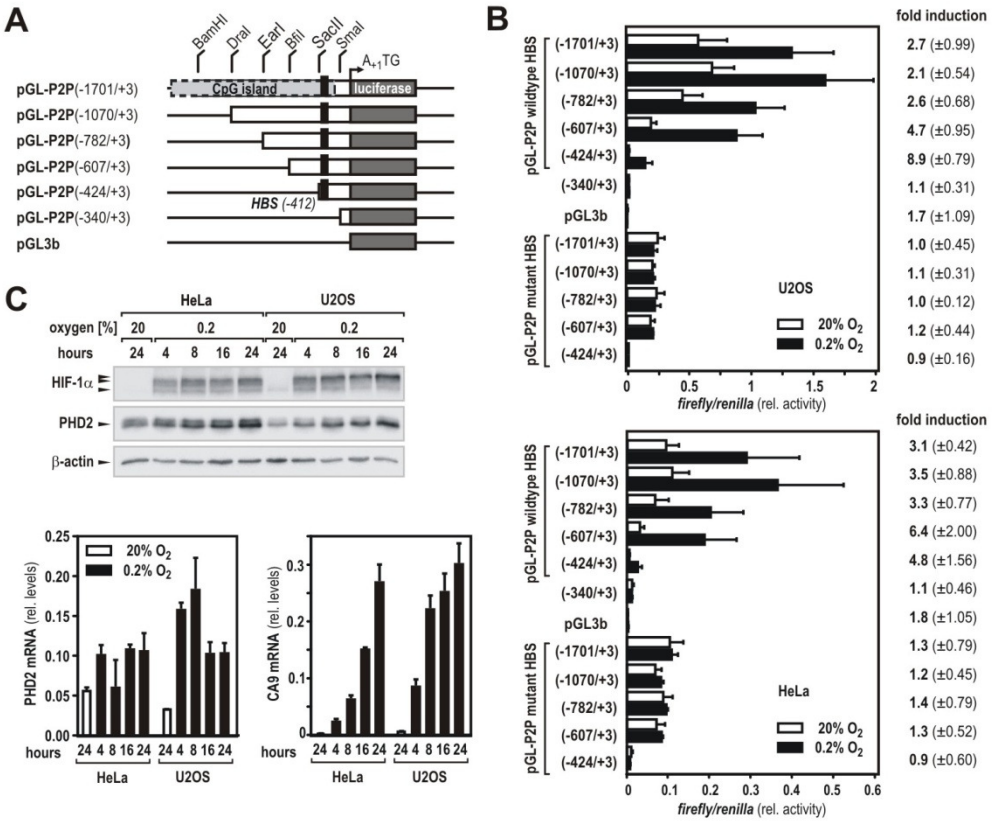

Figure 1. Identification of the minimal human *PHD2* promoter.

(A) Schematic representation of *PHD2* promoter (P2P) 5'-truncations and their cloning strategy as used in this study. The translational start site is designated "+1". **(B)** Regulatory DNA regions of the human *PHD2* gene were cloned into luciferase reporter vectors that were transiently transfected into human U2OS osteosarcoma cells. One day after transfection, cells were incubated for 24 hours at 20% or 0.2% O_2. Hypoxic induction factors (mean values ± S.D.) of relative luciferase activities were calculated from three independent experiments performed in triplicates. Mutation of a single HIF binding site (HBS, black rectangles in *A*) completely abrogated hypoxic inducibility of all constructs. **(C)** Hela and U2OS cells were incubated at 20% or 0.2% O_2 for 4-24 hours and protein levels of HIF-1α, PHD2 and β-actin were analyzed by immunoblotting. Total RNA was isolated from cultures treated as in *B* and mRNA levels of PHD2 and carbonic anhydrase 9 (CA9) were determined by RT-qPCR. Transcript levels of CA9 served as positive control to confirm continuous hypoxic responses. Gene expression levels were expressed in relation to ribosomal L28 mRNA (rel. levels) calculated from 3 independent experiments (±S.D.).

3.2.2 A differential screening approach to identify site-specific transcription factor interplay

Multiplexed transfection of *firefly* and *renilla* reporter genes controlled by either wildtype or HBS-mutant *PHD2* promoters, respectively, allows to classify any reporter-modulating event as HIF-dependent or self-sufficient. Moreover, the dual reporter system provides a read-out that permits to screen under HIF-stabilizing conditions (e.g. hypoxia) while simultaneously assessing the non-induced background levels (simulated “normoxia”) in the very same cells, thereby reducing intra-assay variabilities and screening complexity. Nucleotides -607 to +3 of the *PHD2* promoter containing either the wildtype or mutant HBS were used to drive transcription of *firefly* or *renilla* luciferase cDNAs, respectively. The two luciferase reporter genes showed identical hypoxic responses in the respective reporter vectors **(fig. 2A)**. As a proof of principle, this system was tested by co-expressing *mlpas*, encoding an inhibitory isoform of HIF-α, with the two reporter plasmids as described above[19]. Indeed, a marked downregulation of hypoxic *firefly* versus *renilla* luciferase activity was observed when compared to co-transfection with the empty vector or an unrelated transcription factor (mHes-1). As expected, forced expression of PHD2 or PHD3 strongly attenuated the hypoxic activation of the reporter system, confirming that also post-translational mechanisms impairing the activation state of the HIF-pathway can be assessed by this method **(fig. 2B)**[17].

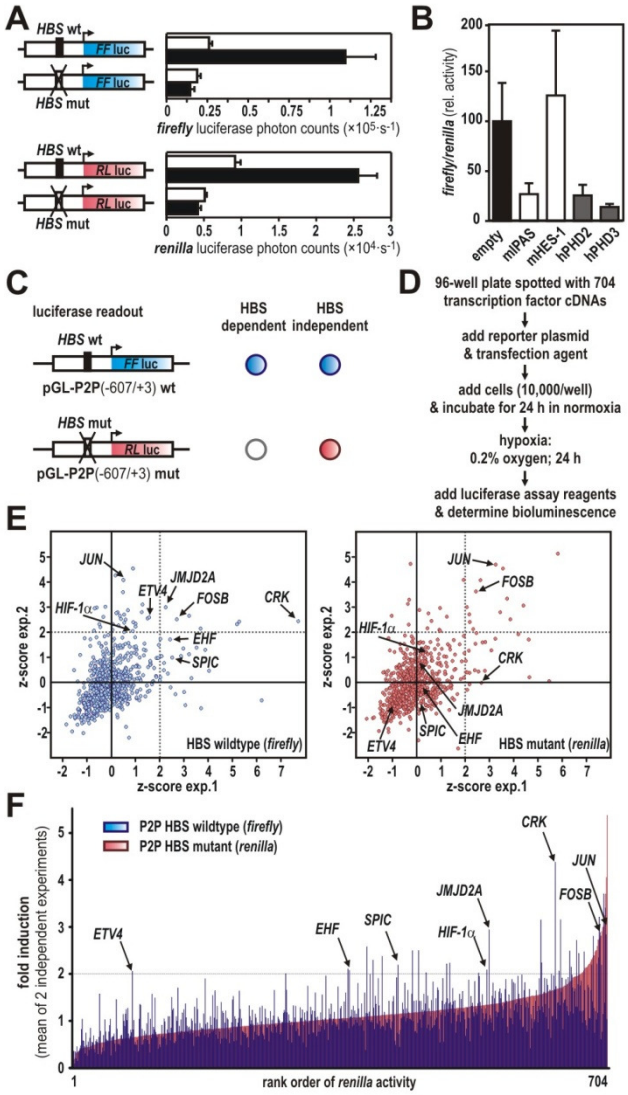

Figure 2. Synthetic transactivation screening allows to discriminate HBS-dependent and independent promoter activation.

(A) P2P(-607/+3) driven *firefly* (blue) and *renilla* (red) luciferase reporter genes transfected into U2OS cells show similar hypoxic activation (0.2% O_2 for 24 hours, black bars) when compared to normoxic (20% O_2, open bars) cultures. HBS mutation completely abrogated hypoxic induction of both constructs. **(B)** Co-expression of transcription factors (mIpas, mHes-1) or PHD enzymes with P2P(-607/+3)wt HBS driven *firefly* and P2P(-607/+3)mut HBS driven *renilla* luciferase reporters, respectively. Dual luciferase activities were determined after 24 hours of hypoxic exposure (0.2% O_2) and expressed as relative luciferase activity normalized to controls receiving the empty expression vector. **(C)** Schematic overview of the *PHD2* promoter-luciferase constructs used for synthetic transactivation screening. Expected readouts for HBS-dependent (*firefly* reporter activation only, blue circle) and independent reporter gene activation (simultaneous activation of *firefly* and *renilla* reporter genes indicated by blue and red circles, respectively) are illustrated by a cross table. **(D)** Workflow of synthetic transactivation screening analyzing a commercial transcription factor expression library. **(E)** Z-scores of two independent screening experiments were plotted for both reporters. Thresholds for z > 2 are indicated by dotted lines. **(F)** Paired values for individual co-expressed cDNAs for *firefly* (blue)

and *renilla* (red) read-outs are depicted. A fold of induction greater than 2 was considered as lead for a re-screening. Positions of HIF-1α and seven new transcription factors positively re-evaluated at a secondary screening level are indicated. Data are given as the mean of two independent screening experiments. For calculations and full gene names refer to the text.

3.2.3 Members of the FOS and ETS transcription factor families transactivate the human *PHD2* promoter

An array of 704 cDNA expression vectors representing all commonly known transcription factor families was screened using the HBS-specific synthetic transactivation readout. Possible HIF co-regulators alter *firefly* but not *renilla* luciferase expression, whereas HIF-independent factors interfere with both reporters (schematically depicted in **fig. 2C**). A work flow of the transfection and screening procedure is given in **figure 2D**. Two independent screens were performed and reproducibility was visualized by plotting z-scores **(fig. 2E)**. Solid correlations were observed for the vast majority of the co-expressed transcription factors, confirming that no significant deviation between the two experiments occurred. Reporter gene inductions by each co-expressed transcription factor were calculated as multiples of the respective 96-well plate median individually for *firefly* and *renilla* activities, averaged over the two experiments, and ranked according to the induction factors of the HBS-mutant *renilla* reporter **(fig. 2F)**. The cut-off was defined as an increase in luciferase activity by a factor of at least 2, tolerating a deviation of 0.05. Factors that showed a reproducible increase in either *firefly* luminescence **(supplementary table S1A)** or *firefly* and *renilla* luminescence together **(supplementary table S1B)** were considered as leads. Underlining the validity of this approach, the only known transcriptional activator of PHD2 expression, namely HIF-1α, was amongst the cDNAs identified **(fig. 2E and F)**. For re-evaluation, the cDNAs of 43 leads were retrieved and partially sequenced to verify their identities. Seven of these cDNAs, namely ETS-variant 4 (ETV4), Spi-C transcription factor (SPIC), ETS homologous factor (EHF), the proto-oncogenes JUN and FOSB, v-crk sarcoma virus CT10 oncogene homolog (CRK) and Jumonji domain-containing protein 2A (JMJD2A), resulted in a reproducible HBS-dependent regulation of the *PHD2* promoter. Most of

these factors can be attributed to two major groups: the ETS (ETV4, EHF, SPIC) and the FOS (JUN, FOSB) families.

3.2.4 ETV4 activates *PHD2* and *transferrin* promoters synergistically with HIF-1

Expression vectors of the seven newly identified factors were co-transfected together with pGL-P2P(-607/+3) HBS wildtype or mutant reporter genes, cloned into identical backbones to exclude false positive effects which may have resulted from the two different luciferase cDNAs in the original screen. In the presence of a functional HBS, co-expression of ETV4 resulted in a striking super-induction of the *PHD2* promoter under hypoxic conditions only **(fig. 3A, left panel)** which was fully lost with the mutant construct **(fig. 3*A*, right panel)**. Since ETV4 was the strongest hit identified, subsequent work focused on the role of this transcription factor as a putative transactivator of PHD2. Of note, the *PHD2* promoter region used in this screen lacks a consensus 5'-$^{A}/_{C}$GGAAGT-3' ETV4 binding site[20]. Thus, direct binding of ETV4 to the *PHD2* promoter appears unlikely, though not fully excluded regarding the small residual stimulation of constitutive *PHD2* promoter activity following HBS mutation. However, the complete lack of hypoxic *PHD2* promoter stimulation by ETV4 suggests that HIF-1 might actually recruit ETV4 to enhance hypoxic induction. Accordingly, knockdown of HIF-1α by RNA interference in U2OS cells abrogated hypoxia and ETV4 mediated induction of the wildtype *PHD2* promoter **(fig. 3B, right panel)**, while an unrelated control siRNA had no effect **(fig. 3B, left panel)**. A heterologous reporter construct driven by the minimal *SV*40 promoter in conjunction with either a wildtype **(fig. 3C, left panel)** or mutant **(fig. 3C, right panel)** hypoxia response element derived from the human *Transferrin* gene recapitulated the strong hypoxic superinduction by exogenous ETV4, proposing a more general model of synergistic interaction between ETV4 and HIF-1[21]. In line with our screening data, overexpressed ETV4 significantly up-regulated endogenous PHD2 mRNA and protein levels in hypoxic U2OS cells, while PHD1 mRNA levels remained unaffected **(fig. 3D and E)**.

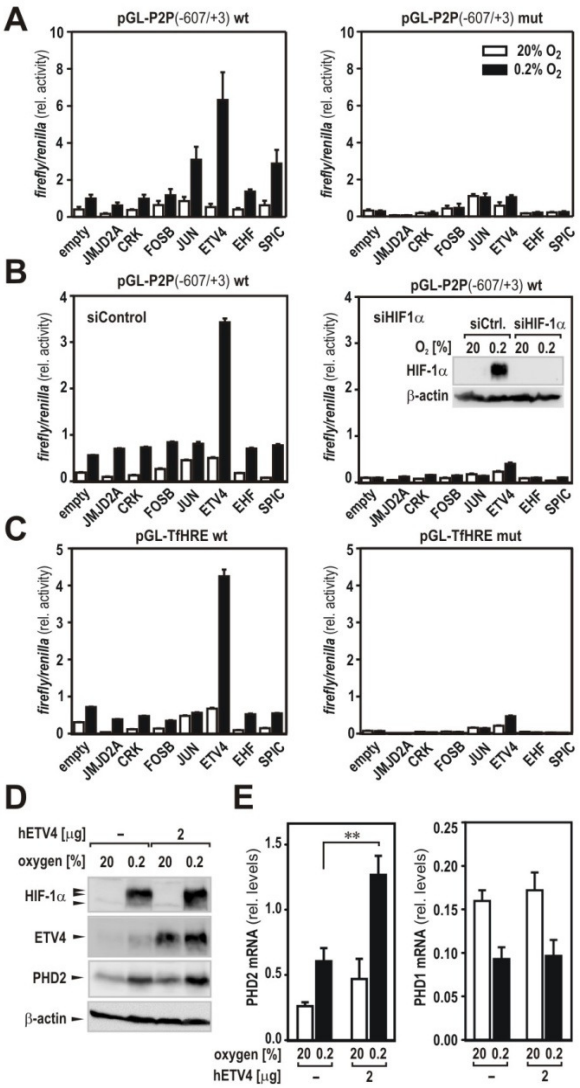

Figure 3. Hypoxic transactivation of the *PHD2* promoter by ETV4 requires HIF-1α activity.

(A) Standard dual luciferase reporter gene assays of seven re-evaluated hits from the transcription factor overexpression array. Wildtype (left panel) or HBS mutant (right panel) *PHD2* promoter regions controlling *firefly* luciferase reporter plasmids were co-transfected into U2OS cells together with expression constructs of the aforementioned factors. Transfection of an empty expression vector (empty) served as negative control and differences in transfection efficiency were controlled by co-transfecting *SV40* promoter driven *renilla* luciferase. Cells were cultured at 20% or 0.2% oxygen for 24 hours before dual luciferase activities were determined. **(B)** Transient RNAi mediated knockdown of HIF-1α fully abrogated hypoxic activation of the *PHD2* promoter by ETV4. U2OS cells were transiently transfected with siRNA oligonucleotides targeting HIF-1α (siHIF1α, right panel) or a control sequence having no human target (siControl, left panel). Reporter gene experiments using the P2P reporter construct with only wildtype HBS were performed as described in *A*. The inset shows an immunoblot confirming the robust knockdown of HIF-1α in U2OS cells. **(C)** ETV4 and HIF-1 synergism in hypoxic gene activation is not restricted to the *PHD2* promoter. A heterologous hypoxia

responsive reporter gene containing two functional HBS from the human *Transferrin* hypoxia response element (pGL-TfHRE wt) was tested in luciferase reporter assays as described in *A*. Mutation of both HBS (pGL-TfHRE mut) caused an abrogation of the signal as seen in *A*. **(D, E)** Forced expression of ETV4 in U2OS cells upregulates endogenous PHD2 protein and transcript levels. **(D)** Whole cell lysates were prepared from cells exposed for 16 hours to 20% or 0.2% oxygen and analyzed for HIF-1α, ETV4, PHD2 and β-actin levels by immunoblotting. **(E)** Total RNA was extracted of similarly treated cells and mRNA levels of PHD1, PHD2 and L28 were quantified by RT-qPCR. Data are shown in relation to ribosomal L28 mRNA (rel. levels) calculated from 3 independent experiments (**p<0.01, paired *Student's* t-test).

3.2.5 ETV4 interaction with the carboxy-terminal transactivation domain of HIF-1α depends on co-recruitment of p300

Intrigued by the HIF-dependent ETV4 effects, we aimed for the characterization of a putative physical interaction between ETV4 and HIF-1α. Utilizing a mammalian two-hybrid system, expression plasmids encoding for ETV4 fused to the activation domain (AD) of viral protein 16 (VP16-ETV4) were co-transfected with HIF-1α oxygen regulatory domains fused to a Gal4-DNA binding domain (DBD, see **fig. 4A)**[22,23]. Due to its intrinsic transactivation activity, constructs containing the carboxy-terminal activation domain (CAD) of HIF-1α (amino acids 775-826) were sufficient to activate the Gal4-responsive promoter **(fig. 4B)**[23]. Co-expression of ETV4 strikingly superinduced GH1α740-826 and GH1α786-826, particularly under hypoxic conditions, suggesting that HIF-1α CAD and ETV4 co-operate to transactivate target genes **(fig. 4B)**. ETV4 effects on the amino-terminal activation domain (NAD; HIF-1α amino acids 549-582) were negligible[23]. Both, HIF-1α and ETV4 have been demonstrated to interact with the ubiquitous transcriptional co-activators p300/CBP[24,25]. To address the question whether the two factors directly interact or whether a ternary complex between HIF-1, p300/CBP and ETV4 is formed (schematically depicted in **fig. 4C**), binding of HIF-1α CAD to p300 was disrupted by forced overexpression of CBP/p300-interacting transactivator 2 (CITED2), known to negatively regulate HIF function[26]. Structural analyses revealed that CITED2 and HIF-1α share an overlapping binding interface in the p300 cysteine-histidine-rich 1 (CH1) domain and competition assays showed a 33-fold higher affinity of CITED2 for

binding to p300 CH1 than a corresponding HIF-1α CAD peptide, indicating that CITED2 is a dominant inhibitor of HIF-1α:p300/CBP complex formation[27]. HIF-1α CAD:ETV4 interplay was totally abrogated by CITED2 in mammalian two-hybrid experiments **(fig. 4D)**, underscoring the assumption that ETV4 co-activation of HIF-1 requires functional interaction of the latter with p300/CBP.

While transient knockdown of p300 slightly reduced the intrinsic transactivation activity of GH1α786-826 in mammalian two-hybrid experiments, robust superinduction of this construct occurred when VP16-ETV4 was co-transfected, irrespective of the presence of p300 **(fig. 4E)**. Thus, we assume that both p300 and CBP can function as bridging molecules for HIF-1α CAD:ETV4 interplay. Underlining the essential requirement of p300/CBP for HIF-1α:ETV4 interaction, normoxic transactivation activity of co-transfected GH1α786-826 and VP16-ETV4 was similar to hypoxic activation levels in U2OS cells transiently depleted of FIH **(fig. 4F)**. FIH has been described previously as oxygen-dependent negative regulator of HIF-α:p300/CBP interaction[7]. Chromatin immunoprecipitation (ChIP) experiments using either anti-HIF-1α or anti-ETV4 antibodies revealed oxygen-dependent enrichment of the HRE-containing *PHD2* (*EGLN1*) promoter region in both precipitations **(fig. 4*G*)**, providing further evidence for co-recruitment of the two transcription factors to the endogenous *PHD2* locus.

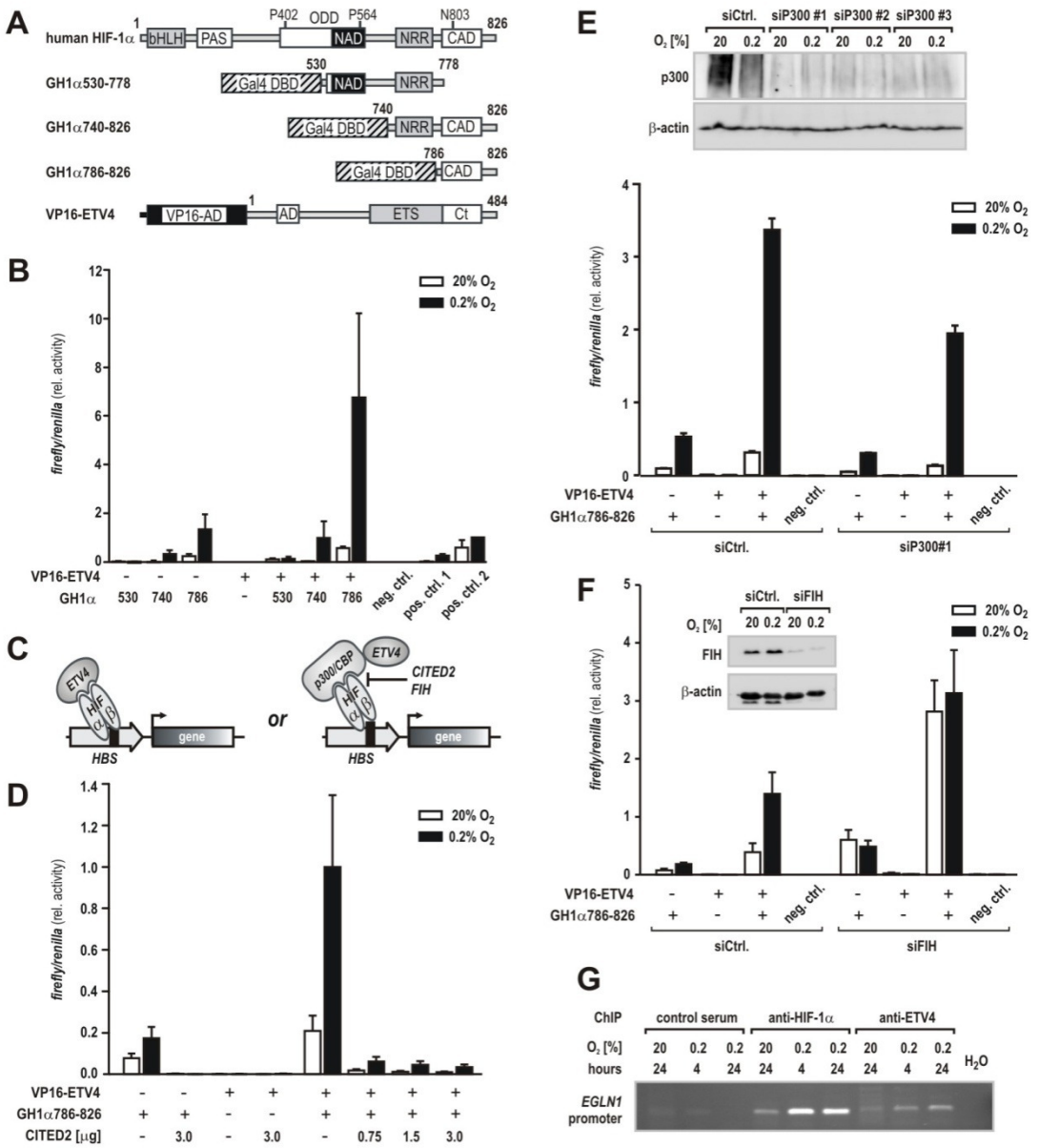

Figure 4. Transcriptional cooperation between ETV4 and HIF-1 is disrupted by CITED2.

(A) Schematic representation of HIF-1α and ETV4 domain structure and fusion constructs used in mammalian two-hybrid assays. PAS, PER-ARNT-SIM; bHLH, basic helix-loop-helix domain; ODD, oxygen-dependent degradation domain; NRR, negative regulatory region; NAD and CAD, amino- and carboxy-terminal activation domains, respectively. A GAL4-DNA binding domain (DBD) was fused to regions encompassing the HIF-1α NAD and CAD. Full-length ETV4 bearing two activation domains (AD, acidic domain; Ct, carboxy-terminal tail) flanking a central ETS domain was fused to a VP16 activation domain (VP16-AD). Numbers indicate the amino acids present in the respective constructs. **(B)** U2OS cells were co-transfected with a Gal4-responsive reporter plasmid and Gal4-HIF-1α (GH1α) constructs alone or in combination with VP16-ETV4. The GH1α fusion constructs are specified by the amino-terminal starting amino acid of the truncated HIF-1α regions (530, 740 and 786, respectively).

Following transfection, cells were evenly split and incubated at 20% or 0.2% O_2 before luciferase activities were determined 24 hours later. Non-interacting Gal4 DBD-p53 and VP16-AD-CP1 served as negative control (neg. ctrl.), while the interactions between Gal4 DBD-PHD2 and VP16-AD-HIF-2α(ODD) or VP16-AD-FKBP38 were used as positive controls (pos. ctrl. 1 and pos. ctrl. 2, respectively). **(C)** Scheme of the potential interactions between HIF-1, p300/CBP and ETV4 as assessed by mammalian two-hybrid assays. Both CITED2 and FIH can block the interaction between HIF-1α and p300/CBP. **(D)** Co-transfection of the indicated amounts of a CITED2 expression construct together with the mammalian two-hybrid expression vectors followed by hypoxic exposure and luciferase activity determination as described for *B*. **(E)** Co-transfection of siRNA directed against p300 together with the mammalian two-hybrid expression vectors followed by hypoxic exposure and luciferase activity determination as described for *B*. The p300 knockdown efficiency of different siP300 oligonucleotides was analyzed by immunoblotting (upper panel) and siP300#1 was chosen for further experiments. **(F)** Co-transfection of siRNA directed against FIH together with the mammalian two-hybrid expression vectors followed by hypoxic exposure and luciferase activity determination as described for *B*. The efficiency of the siFIH mediated FIH knockdown was confirmed by immunoblotting as shown in the inset. **(G)** Chromatin immunoprecipitation (ChIP) of normoxic or hypoxic PC3 cells using antibodies directed against HIF-1α or ETV4, or control serum. The amount of co-precipitated chromatin derived from the human *PHD2* promoter region (encoded by *EGLN1*) containing the HBS was determined by PCR followed by agarose gel electrophoresis.

3.2.6 Both HIF-α isoforms are capable of forming a complex with ETV4

Fluorescence resonance energy transfer (FRET) analyses of co-expressed ETV4 and HIF-1α marked with cyan or yellow fluorescent protein tags (CFP and YFP, respectively) resulted in a robust energy transfer between both factors. Similar FRET efficiencies were observed when YFP-labeled HIF-2α was used together with CFP-ETV4 **(fig. 5A and B)**. The intracellular distance of the two nuclear proteins was calculated to be 5.6-5.7 nm and did not differ in oxygenated or hypoxic cells, which might be explained by saturation of the HIF-α degradation pathways by exogenous overexpression of the transcription factors. Notably, efficient energy transfer between HIF-1α and p300 at ambient oxygen tensions has been reported previously[28].

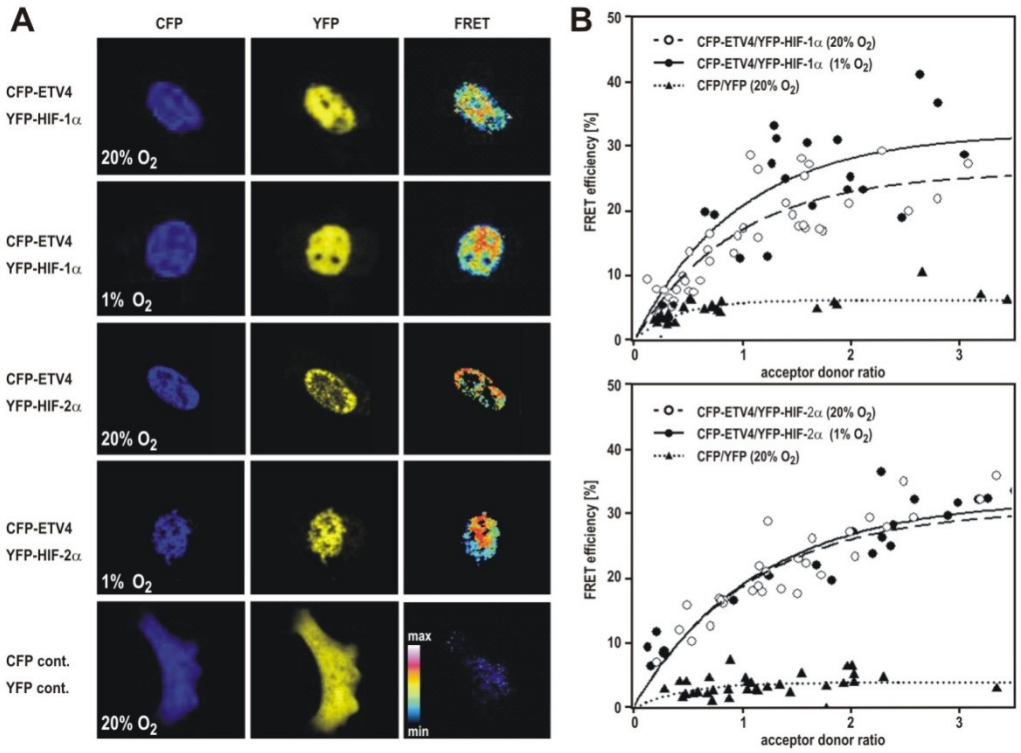

Figure 5. Both HIF-1α and HIF-2α co-localize with ETV4 to the nucleus within molecular proximity.

U2OS cells were transiently transfected with the indicated CFP or YFP plasmids, and FRET analysis was performed at 20% O_2 or 1% O_2 24 - 48 hours post transfection. **(A)** Microscopic images showing the subcellular localization of the exogenous proteins. Fluorescence intensity of FRET signals is visualized by false colors on a color bar from low (blue) to high (white) intensity. **(B)** FRET efficiencies for CFP-ETV4 and YFP-HIF-1α (upper panel) or YFP-HIF-2α (lower panel) fusion protein pairs were calculated from 20 - 40 randomly selected cells which displayed various fluorescent acceptor/donor ratios. Scatter plots were fit to a single-site binding model. FRET efficiencies are given as the percentage of transferred energy relative to the energy absorbed by the donor.

3.2.7 A broad role for ETV4 in hypoxic gene expression

Recent work reported high ETV4 expression levels in the human prostate cancer cell line PC3 that lacks the constitutive photomorphogenic protein COP1 acting as E3 ubiquitin ligase for a variety of ETS proteins[29,30]. Since endogenous ETV4 expression levels in U2OS cells was close to the detection limit, the PC3 cell model was chosen to study the involvement of ETV4 in the hypoxic response by applying a genome-wide expression array screening. PC3 cells lentivirally infected with shRNA expression constructs targeting ETV4 (shETV4) revealed a robust knockdown of mRNA and protein levels, while a non-target control shRNA (shNTC) did not affect ETV4 expression **(fig. 6A and B)**. ETV4 depleted PC3 cells showed robustly reduced mRNA levels of the known ETV4 target gene cyclooxygenase-2 (*COX2*), confirming loss of ETV4 function in these cells **(fig. 6B)**[25,31]. Total RNA was isolated from PC3 shETV4 and shNTC control cells exposed to 20 or 0.2% oxygen for 24 hours and samples from three independent experiments were labeled for microarray analysis. When compared to normoxic control cells, 977 mRNAs and large intergenic non-coding RNAs (lincRNAs) were more than 2-fold downregulated ($p<0.05$) in normoxic PC3 cells lacking ETV4 **(fig. 6C; green)**. Hypoxia alone upregulated 608 mRNAs/lincRNAs more than 2-fold ($p<0.05$) **(fig. 6C; red)**. Interestingly, 450 mRNAs/lincRNAs showed a more than 2-fold reduction ($p<0.05$) of the hypoxic expression levels in cells lacking ETV4 when compared to hypoxic control cells **(fig. 6C; blue)** out of which a group of 47 mRNAs/lincRNAs was found to be simultaneously hypoxia-inducible. Individual expression levels of these 47 transcripts centered on the mean of the three normoxic control samples (PC3 shNTC) are depicted in a heatmap in **figure 6D**. Array data were validated by RT-qPCR of four randomly chosen transcripts **(fig. 6E)**.

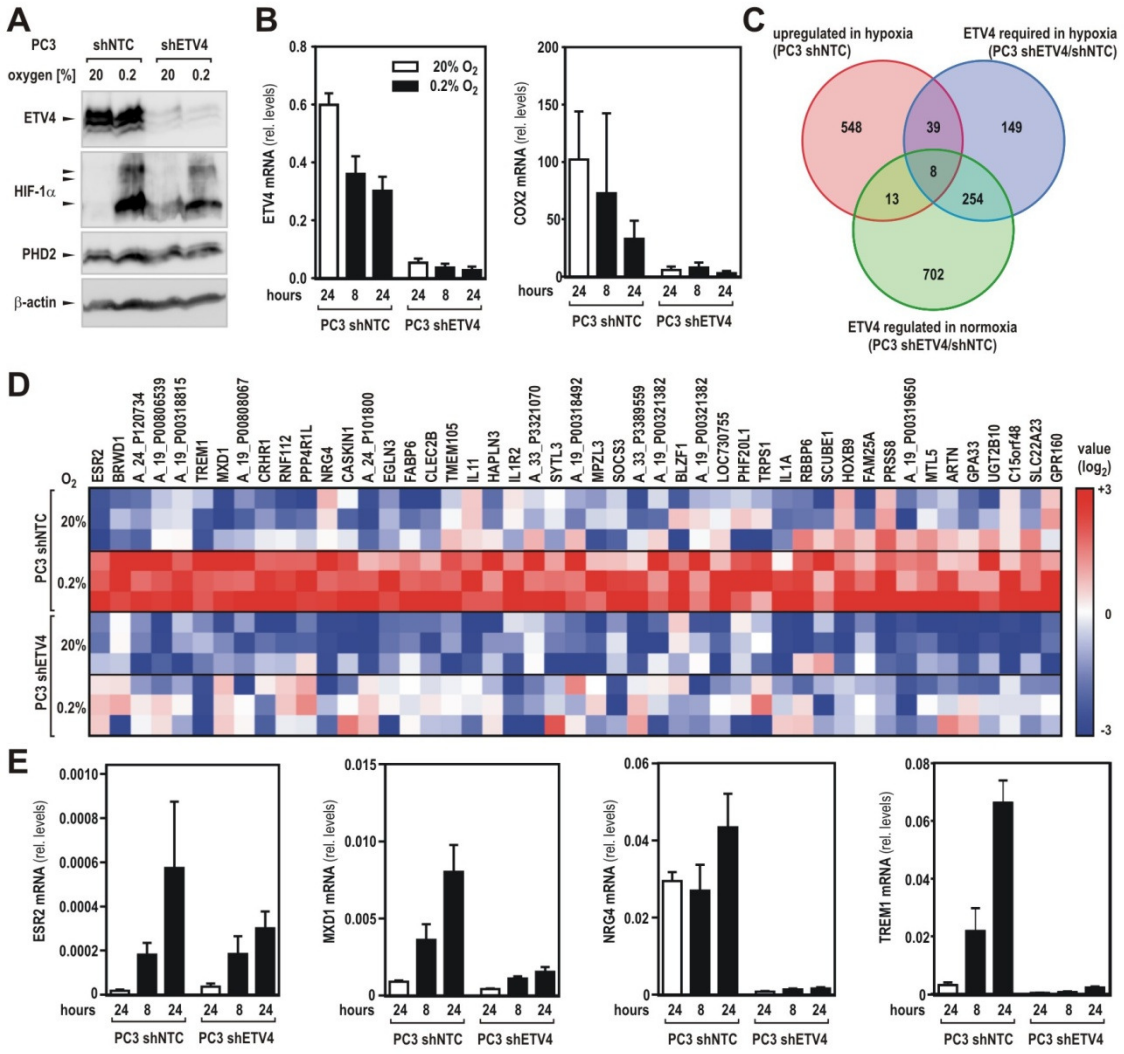

Figure 6. Genome-wide microarray expression analysis reveals a broad role for ETV4 in HIF mediated hypoxic gene regulation.

(A) Efficient knockdown of ETV4 in human PC3 prostate cancer cells. PC3 cells were stably transduced with lentiviral shRNA expression vectors encoding either a non-target control (shNTC) or shETV4. Following 24 hours of exposure to 20% O_2 or 0.2% O_2, ETV4, HIF-1α, PHD2 and β-actin protein levels were analyzed by immunoblotting. **(B)** Total RNA was isolated from cultures treated as in *A* and mRNA levels of ETV4 and its target gene COX2 were determined by RT-qPCR. Gene expression levels were expressed in relation to ribosomal L28 mRNA (rel. levels) calculated from 3 independent experiments. **(C)** Venn diagram showing the number of transcripts regulated by either an at least two-fold induction by hypoxia alone (red), an at least two-fold reduction in normoxic cells by the knockdown of ETV4 (green), or an at least two-fold reduction in hypoxic cells by the knockdown of ETV4 (blue), respectively. **(D)** Heatmap of the individual expression levels of the 47 transcripts that required ETV4 for efficient hypoxic induction. **(E)** Expression levels of four randomly chosen transcripts shown in *D* were confirmed by RT-qPCR as described for *B*.

3.2.8 HIF target genes divide into two groups, either ETV4 co-activated or independent

Because hypoxically upregulated gene sets are highly variable between different cellular models, we next focussed on a pre-defined gene set of 61 hypoxia-inducible transcripts. This gene set has previously been reported based on established HIF target genes[32]. In line with this publication, the majority of these genes was found to be hypoxically upregulated in PC3 shNTC control cells (green dots in **fig. 7A**, **left panel**). Comparing hypoxic expression levels of the same genes in PC3 shETV4 knockdown with shNTC control cells, the group of established HIF target genes roughly clustered into two halves, representing transcripts which either remained unaffected or which did not respond to hypoxia anymore in the absence of ETV4 **(fig. 7A, right panel)**. Interestingly, following ranking of the HIF target genes according to their requirement for ETV4, the HIF-dependent PHD3 oxygen sensor (encoded by *EGLN3*) showed the highest ETV4 sensitivity for hypoxic induction in PC3 cells **(fig. 7B and C)**, demonstrating that ETV4 plays a major role in the feedback control of mammalian oxygen sensing. However, a number of established HIF-responsive genes was only slightly affected (e.g. PAI1, encoded by *SERPINE1*) or completely resistant (e.g. GLUT1, encoded by *SLC2A1*) to ETV4 depletion **(fig. 7B and C)**, suggesting that the HIF pathway can be divided into two branches according to the requirement for ETV4.

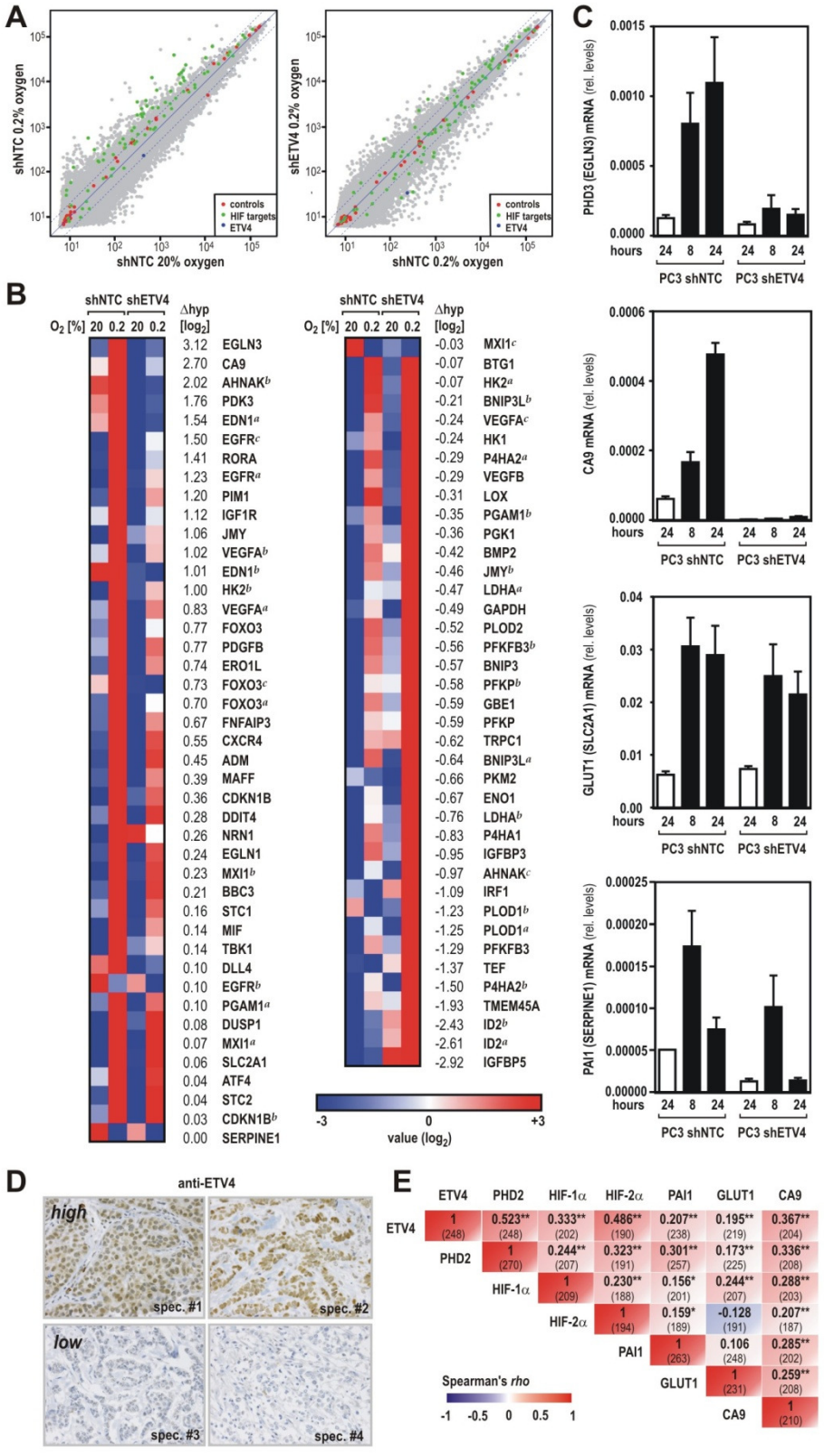

Figure 7. Role of ETV4 in the regulation of established HIF target genes *in vitro* and *in vivo*.

(A) Dot plots showing the correlation between transcripts in normoxic vs. hypoxic control cells (left panel) or in hypoxic control vs. hypoxic ETV4 knockdown cells (right panel) as derived from the gene array data (grey dots). Red dots refer to internal controls and the blue dot shows ETV4 which is

downregulated in shETV4 cells. Green dots indicate the positions of a pre-defined set of 61 well-established HIF target genes. **(B)** Heat map of the 61 HIF target genes ranked by the magnitude of ETV4 requirement for hypoxic induction according to differences in hypoxic expression levels with $\Delta hyp = \log_2(shNTC\_hypoxia) - \log_2(shETV4\_hypoxia)$ and mean hypoxic expression levels centered to the mean of normoxic control cells. **(C)** Exemplary mRNA levels of HIF target genes which either require ETV4 for efficient hypoxic induction (PHD3 and CA9) or which remain unaffected by the ETV4 knockdown (GLUT1 and PAI1). mRNA was quantified as described for Figure 6*B*. **(D, E)** Correlation between ETV4 and established markers for tissue hypoxia in human breast cancer. **(D)** Independent specimens (spec.) of immunohistochemical evaluation of ETV4 expression in primary mammary carcinoma with high (upper panel) or low (lower panel) ETV4 expression levels. **(E)** Rank-order correlations (Spearman's *rho*) for ETV4 and PHD2 as well as known markers reflecting tissue hypoxia (HIF-1α, HIF-2α, PAI1, GLUT1 and CA9) are summarized in a cross table. The number of cases where both of the correlated markers could be assessed is displayed in parentheses. Asterisks indicate statistical significance with *$p<0.05$ and **$p<0.01$.

3.2.9 ETV4 expression levels correlate with HIF-α accumulation in human breast cancer

Elevated ETV4 transcript levels have been reported in a variety of human neoplastic diseases including breast cancer[33]. Moreover, the onset of spontaneous mammary tumor development has been shown to be profoundly delayed in MMTV-*neu* transgenic mice which express a dominant negative variant of the mouse homologue of ETV4, suggesting that ETV4 may possess tumor promoting effects[34]. We recently characterized protein levels of a variety of hypoxic marker genes, including HIF-1α, in tumor samples of 282 patients diagnosed with primary breast carcinoma[13,35]. When the same entities were immunostained for ETV4 protein levels, a strong and highly significant correlation between ETV4 and PHD2 was observed in samples where both factors were assessed ($p<0.01$, Spearmans's *rho*, N=243). Consistent with our model of synthetic action of ETV4 and HIF-α, a solid association was also observed between these transcription factors and three well described target genes (PAI1; GLUT1; CA9) of the HIF pathway. Correlation coefficients between ETV4, PHD2 and HIF-1α and HIF-2α were considerably higher than those observed amongst both HIF-αs and the three markers for tissue hypoxia **(fig. 7D and E)**, demonstrating a putative role for ETV4 in the regulation of PHD2 expression also *in vivo*.

3.3 Discussion

Unique responsiveness to altered oxygen environments and broad conservation of all components of the PHD/HIF oxygen sensing pathway in multicellular life clearly indicate the central role of HIFs as literally *hypoxia-inducible* transcription factors[36]. Eukaryotic gene expression, however, is a multi-step process requiring the complex transcription machinery to interact with promoter DNA and initiate transcription[37]. Not surprisingly, numerous studies have identified other nuclear regulators that contribute to the full spectrum of transcriptional changes in response to hypoxia. Yet, general patterns of direct interplay amongst HIFs and other transcriptional regulators are largely unknown and interactions were often found to rely on specific cell models[38]. Here, we report on a novel screening approach that, in combination with overexpression of arrayed transcription factors, aimed for the systematic analysis of HBS-specific transcription factor interplay. The core *PHD2* promoter was employed as a paradigm for HIF-dependent gene regulation, since it embeds a single HBS conferring hypoxic activation and because the endogenous locus is ubiquitously expressed.

We identified various members of the activating protein-1 (AP-1) family as novel activators of the *PHD2* gene, though JUN/FOS have been found to enhance hypoxic gene expression previously[38]. However, to the best of our knowledge our study is the first to link ETV4 with HIF-dependent transcription. As shown by the use of different reporter genes, ETV4 function as facilitator of HIF-1 transactivation activity is not restricted to the *PHD2* promoter. While nuclear distances between ETV4 and HIF-1/2α, as calculated by FRET experiments, support a close interaction, our data favor a model where p300/CBP serves as essential bridging molecule between the two factors. This conclusion is based on the following features of the interaction between ETV4 and HIF-1α: *i.* oxygen sensitivity in the absence of the oxygen-dependent degradation domain; *ii.* mapping to the C-terminal activation domain; *iii.* competition by CITED2; and *iv.* requirement of FIH for oxygen sensitivity. Such a ternary complex is still in line with the FRET data, as previous findings suggest binding of HIF-1α to both CH1 and CH3 domains of p300[39]. ETV4 is known to interact with p300 at its CH3 domain and thus might well get into close or even physical contact to HIF-1α[40]. Interestingly, out of the 371 known interactors of human p300/CBP, 101

(27.2%) were present in the synthetic transactivation screen, but only 10 (9.9%) of them met the criteria to be considered as reproducible activators of the *PHD2* promoter[41]. Apparently, there is no simple redundancy amongst the p300/CBP interactors to serve as transcriptional co-activators, and the target gene context is thought to play an important role in p300/CBP complex formation[41]. The latter is of particular importance, as it provides some reliability regarding the specificity of our screening approach, underlining its general applicability.

Based on literature searches, ETV4 and HIF pathways share several common target genes, including matrix metalloproteases (MMP) 1, MMP-3, MMP-7, MMP-9, iNOS and COX-2[42–45]. Hence, one might speculate that the two factors directly cooperate at regulatory elements of these genes, jointly boosting invasive properties of malignant cells. Notably, ETV4 and its close relatives ETV1 and ETV5 are frequently overexpressed in prostate cancer due to gene fusion with androgen-responsive gene *loci*[46]. Similarly, loss of the tumor suppressor PTEN causes high normoxic expression levels of HIF-1α, a key feature of invasive prostate cancers[47]. Recent studies employing animal models propose a mutational sequence, where early loss of PTEN and overactivation of ETS target genes collectively promote prostatic cancer progression[30,48]. Thus, it will be highly interesting to explore a putative synthetic HIF-1/ETV4 role in these pathologies.

Due to its high endogenous expression levels in the PC3 prostate cancer cell line, gene array analyses in ETV4 wildtype and knockdown PC3 cells were undertaken to explore the general role of ETV4 in hypoxic gene regulation. Remarkably, 47 of 608 hypoxically induced transcripts depend on ETV4 for efficient upregulation. Further analysis concentrating on a set of 61 established HIF target genes revealed 14 genes whose hypoxic induction was at least two-fold higher in the presence of ETV4 than in its absence. For example, hypoxic induction of carbonic anhydrase 9 (*CA9*) was strongly dependent on the presence of ETV4, as it was largely absent in ETV4 knockdown cells. This finding further explains the unusually strong hypoxic inducibility of *CA9* which has previously been attributed to the cooperation between HIF and ATF-4, another transcription factor that we found to be involved in oxygen signaling[49,50]. Somewhat unexpected, PHD2 did not fulfill, at least in PC3 cells, our

stringency criteria for ETV4-dependent hypoxically induced genes. Individual inspection revealed a 2.7-fold hypoxic induction that was only reduced by 16% in the absence of ETV4. Low hypoxic inducibility in this cell type was also seen on the protein level **(fig 6A)** and might explain the rather weak response of this gene to ETV4 depletion, despite the fact that forced expression of ETV4 strongly induced the *PHD2* promoter in the U2OS cell model. However, out of the 61 established HIF target genes, PHD3 was most sensitive to ETV4 depletion, suggesting that in PC3 cells PHD3 rather than PHD2 might represent the primary oxygen sensor targeted by ETV4.

In vivo, ETV4 has been implicated in kidney branching morphogenesis, differentiation of spinal motor neurons and mammary gland development[51–54]. Importantly, ETV1 (ER81), ETV4 (PEA3/E1AF) and ETV5 (ERM) are highly similar and constitute the PEA3 sub-family among the ETS-domain family of transcription factors[55]. ETV4 and ETV5 are functionally highly redundant and a double knockout was required to reveal the role of ETV4 in kidney development[51,52]. Thus, we tested the ability of these additional sub-family members to induce the *PHD2* promoter. Whereas ETV1 did not have any effect, ETV4 and ETV5 similarly super-induced the hypoxic PHD2 and transferrin promoters in a HBS-dependent manner, suggesting functional redundancy of these two sub-family members in hypoxic gene regulation **(suppl. fig. S1)**. Since the DNA-binding/ETS domain is highly conserved between all three PEA3 sub-family members, we further conclude that the interaction with HIFα takes place outside of the ETS domain of ETV4.

Because these developmental processes often occur in tissues with low oxygenation, our data point to a role of tissue hypoxia in physiological ETV4/ETV5 function. Likewise, high expression levels of ETV4 have been linked to metastasis or bad prognosis in a variety of human cancers[56]. These clinical features are well known for hypoxic tumors expressing high levels of HIF-1[57]. Supporting our screening results, we found a strikingly good correlation between ETV4 and PHD2 protein levels in breast cancer tissues, in line with a potentially relevant function of ETV4 in hypoxic tissues *in vivo*.

In summary, synthetic transactivation screening as exemplarily demonstrated for HIF-dependent gene expression proved to be a powerful tool to unravel novel interactions amongst common signaling pathways. The general setup of this method may be easily adapted to study other transcriptional pathways. A multiplexed single-well readout system predestines this approach for extensive screening projects, including small molecule library analyses and genome-wide gene silencing approaches, where inter-well variances are technically difficult to control.

3.4 Supplementary Data

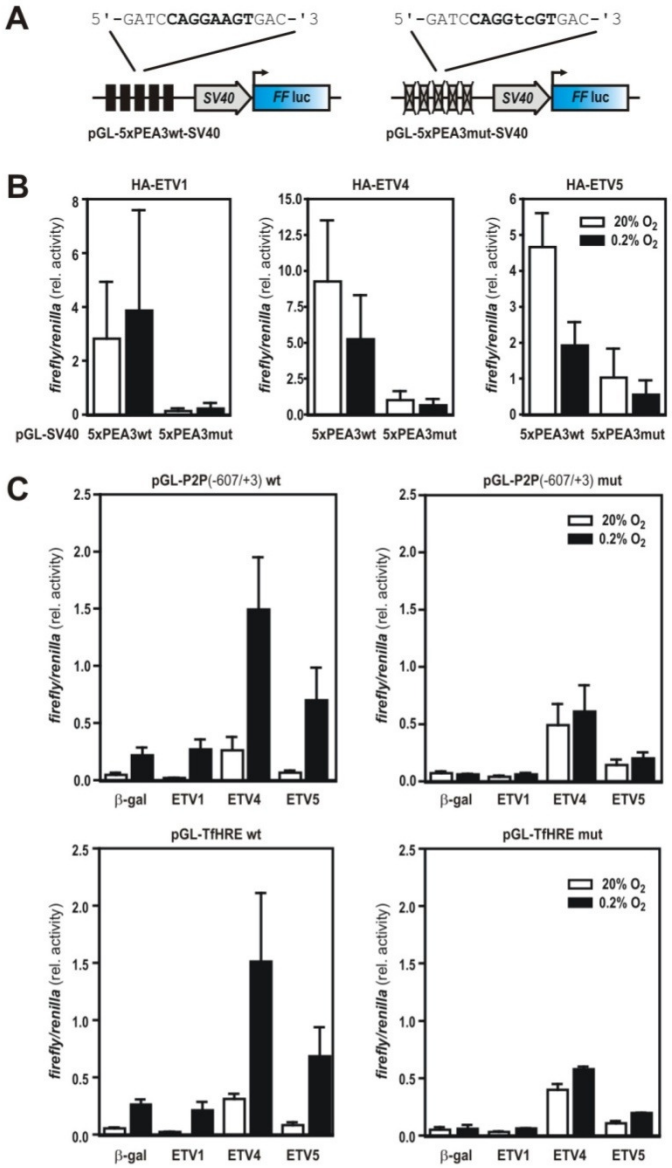

Figure S1. Co-activation of HIF-dependent promoters by the ETS-domain PEA3 sub-family members ETV1, ETV4 and ETV5.

(A) Schematic representation of the SV40 promoter driven *firefly* luciferase reporter gene control constructs containing five PEA3 wildtype (left panel) or mutant (right panel) consensus DNA-binding motifs. **(B)** The *pGL-5xPEA3wt-SV40* or *pGL-5xPEA3mut-SV40* control constructs were co-expressed together with HA-tagged ETV1, ETV4 or ETV5 expression constructs and a *renilla* luciferase control vector in U2OS cells. After transfection, cells were incubated for 16 hours under 20% or 0.2% O_2 before luciferase activities were determined. Shown are ratios of *firefly* to *renilla* luciferase activities of three indpendent experiments performed in triplicates (mean values ± S.D.). All three PEA3-family members activate the wildtype but not the mutant PEA3 consensus motif in a largely oxygen-independent manner. **(C)** *Firefly* luciferase expression vectors driven by the *PHD2* promoter (pGL-P2P(-607/+3); upper panel) or the heterologous SV40 promoter containing two HBS from the human

Transferrin gene (pGL-TfHRE; lower panel) were co-transfected into U2OS cells together with expression constructs for β-galactosidase or HA-tagged ETV1, ETV4 or ETV5, and a *renilla* luciferase control vector. Hypoxic exposure and luciferase determination was as described in *B*. ETV4 and to a somewhat lesser extent ETV5 but not ETV1 superinduced both reporter gene constructs in a HBS-dependent manner.

Table S1A. Candidate factors activating the human *PHD2* promoter in a HBS-dependent manner.

The table summarizes all leads derived from the primary synthetic transactivation screening with gene products inducing preferentially pGL-P2P(-607/+3)wt. Induction factors for *firefly* (*FF*) and *renilla* (*RL*) luciferase values were expressed relative to the respective plate median. Data represent the mean of two independent screening experiments and are ranked according to their ability to activate pGL-P2P(-607/+3)wt by a factor of at least 1.95.

Symbol	fold of median *FF*	fold of median *RL*	Accesion number	Description
CRK	4.38	1.66	NM_005206	v-crk sarcoma virus CT10 oncogene homolog (avian), transcript variant I
ZNF447	3.16	1.71	NM_023926	zinc finger and SCAN domain containing 18 (ZSCAN18)
LHX4	3.15	1.57	NM_033343	LIM homeobox 4
JMJD2A	2.94	1.35	NM_014663	jumonji domain containing 2A
TERF2IP	2.58	1.10	NM_018975	telomeric repeat binding factor 2, interacting protein
SSRP1	2.50	1.18	NM_003146	structure specific recognition protein 1
PBX2	2.50	1.19	NM_002586	pre-B-cell leukemia homeobox 2
FOSL2	2.49	1.85	NM_005253	FOS-like antigen 2
NONO	2.38	1.13	NM_007363	non-POU domain containing, octamer-binding
ZNF323	2.29	1.11	NM_030899	zinc finger protein 323, transcript variant 1
FOXM1	2.24	1.57	NM_021953	forkhead box M1, transcript variant 2
ZNF558	2.23	1.26	NM_144693	zinc finger protein 558
TFEB	2.21	1.25	NM_007162	transcription factor EB
SPIC	2.19	1.15	NM_152323	Spi-C transcription factor (Spi-1/PU.1 related)
FUSIP1	2.15	1.86	NM_006625	FUS interacting protein (serine/arginine-rich) 1, transcript variant 1
FOS	2.11	1.06	NM_005252	v-fos FBJ murine osteosarcoma viral oncogene homolog
HIF1A	2.09	1.35	NM_001530	hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor), transcript variant 1
EHF	2.08	1.07	NM_012153	ets homologous factor
ZNF398	2.06	1.70	NM_020781	zinc finger protein 398, transcript variant 2
ETV4	2.05	0.65	NM_001986	ets variant gene 4 (E1A enhancer binding protein, E1AF), transcript variant 1
SFPQ	2.03	1.21	NM_005066	splicing factor proline/glutamine-rich (polypyrimidine tract binding protein associated)
MGC41917	2.02	1.33	NM_153231	zinc finger protein 550

ZNF200	2.00	0.96	NM_003454	zinc finger protein 200, transcript variant 1
ZNF282	2.00	1.65	NM_003575	zinc finger protein 282
NEUROD1	1.99	1.10	NM_002500	neurogenic differentiation 1
GTF2B	1.99	1.49	NM_001514	general transcription factor IIB
TBPL1	1.97	1.87	NM_004865	TBP-like 1
ZNF606	1.96	1.03	NM_025027	zinc finger protein 606
ZNF43	1.96	1.65	NM_003423	zinc finger protein 43
GABPA	1.95	1.47	NM_002040	GA binding protein transcription factor, alpha subunit 60kDa

Table S1B. Candidate factors activating the human *PHD2* promoter in the absence of a functional HBS.

The table summarizes all leads derived from the primary synthetic transactivation screen with gene products inducing preferentially pGL-P2P(-607/+3)mut. Induction factors for *firefly* (*FF*) and *renilla* (*RL*) luciferase values were expressed relative to the respective plate median. Data represent the mean of two independent screening experiments and are ranked according to their ability to activate pGL-P2P(-607/+3)mut.

Symbol	fold of median *FF*	fold of median *RL*	Accesion number	Description
JUN	2.84	4.06	NM_002228	jun oncogene
EBF1	3.41	3.71	NM_024007	early B-cell factor 1
ZNF655	3.70	3.20	NM_138494	zinc finger protein 655, transcript variant 1
TCEB3	2.19	2.91	NM_003198	transcription elongation factor B (SIII), polypeptide 3 (110kDa, elongin A)
FOSB	2.89	2.81	NM_006732	FBJ murine osteosarcoma viral oncogene homolog B, transcript variant 1
TFDP1	3.21	2.76	NM_007111	transcription factor Dp-1
NR0B2	2.34	2.74	NM_021969	nuclear receptor subfamily 0, group B, member 2
CEBPB	2.21	2.59	NM_005194	CCAAT/enhancer binding protein (C/EBP), beta
SF1	2.81	2.39	NM_201998	splicing factor 1, transcript variant 3
C1orf83	2.19	2.32	NM_153035	chromosome 1 open reading frame 83
TBX5	2.47	2.10	NM_000192	T-box 5, transcript variant 1
MORF4L2	2.43	1.99	NM_012286	mortality factor 4 like 2
ZNF508	2.71	1.96	NM_014913	ADNP homeobox 2

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Conflict of interest statement. None declared.

3.5 Unpublished supplementary data

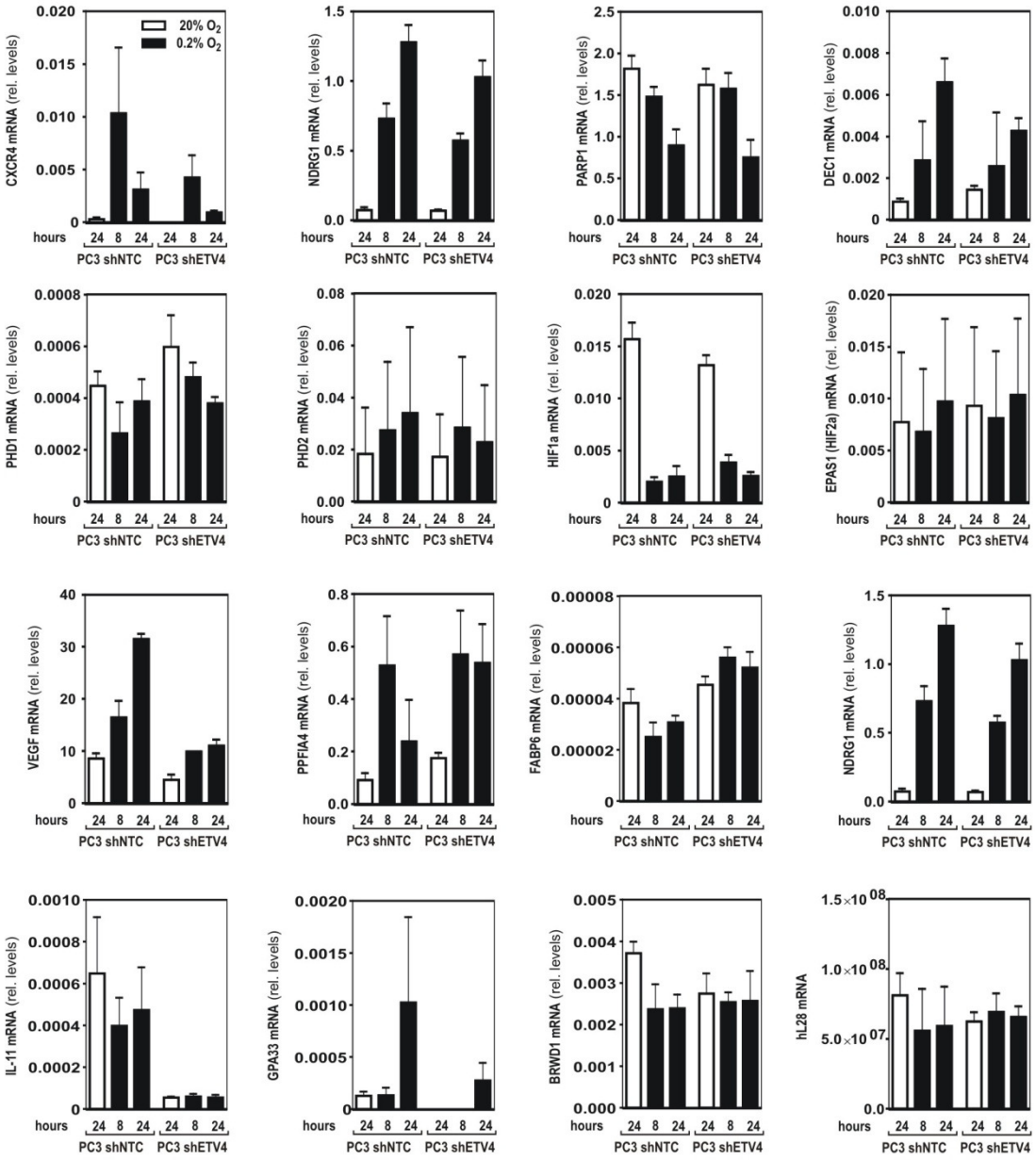

Figure S2. Role of ETV4 in the regulation of established HIF target genes *in vitro*.

(A) A panel of exemplary mRNA levels of HIF target genes which either show tendency to require ETV4 for efficient hypoxic induction (VEGF) or which remain unaffected by the ETV4 knockdown (NDRG1 etc.). mRNA was quantified as described for Figure 6*B*. Measured from three independent experiments except for PHD1 and VEGF that was measured from two independent experiments.

3.6 Materials and methods

Cell culture

Human HeLa cervix carcinoma and U2OS osteosarcoma cells were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma). Human PC3 prostate cancer cells were cultured in Roswell Park Memorial Institute medium (RPMI-1640, Sigma). Media were supplemented with 10% fetal calf serum (FCS) and antibiotics (penicillin 50 IU/ml and streptomycin 100 µg/ml; Gibco-BRL). Hypoxic cell culture was carried out at 0.2% O_2 (if not indicated differently) using a gas-controlled Invivo$_2$ 400 workstation (Ruskinn Technologies). Transfections were performed using polyethylenimine (Polysciences) as described before[17].

PHD2 promoter constructs

PHD2 promoter (P2P) constructs containing the wildtype and mutant HBS in the pGL3basic luciferase vector (Promega) were generated in earlier work[16]. Serial 5'-truncations of P2P and a start codon fusion to the luciferase open reading frame (ORF) were employed for both promoter versions using standard cloning techniques. Within the scope of the screening approach, the *firefly* reporter gene of pGL-P2P(-607/+3) variants was replaced with the *renilla* luciferase ORF cloned into NcoI and XbaI sites.

Transfection and synthetic transactivation screening

Reverse transfection and screening was carried out by using an arrayed expression library containing 704 transcriptionally relevant human full-length cDNAs from the Origene collection (FTCW 19603, GFC-Transfection Array in a 96-well format)[58]. An annotated list of all genes covered by this array is provided online by the manufacturer. Dried DNA (100 ng) of a distinct expression construct spotted per well was reconstituted at room temperature with 20 µl of serum-free medium containing a mixture of pGL-P2P(-607/+3) HBSwt *firefly* and pGL-P2P(-607/+3) HBSmut *renilla* reporter plasmids (100 ng DNA/each). Subsequently, 20 µl of diluted TransIT-LT1 transfection reagent (3:1, µg DNA/ µl TransIT-LT1; Mirus Bio LLC) were added and complex formation was allowed for 30 minutes at room temperature before 60 µl of a cell suspension containing $1x10^4$ U2OS cells in DMEM supplemented with 10% FCS

were plated in each well. Plates were incubated at 20% O_2 for 24 hours before being subjected to the screening conditions of 0.2% O_2 for an additional 24 hours. Cultures were lysed in 20 µl of passive lysis buffer (Promega) and luminescence was immediately analyzed with a microplate luminometer (Berthold) using a standard dual luciferase reporter assay system (Promega). Luciferase activities were normalized to the median calculated individually for each plate and luminescence source and expressed as induction factors (IF) according to ($IF = \frac{N_i}{median_i}$) with N_i = individual luciferase activity value of each well of plate *i* and $median_i$ = median of luciferase activities of all 96-wells on plate *i*. To compare the distribution of replicate assays a standard z-score evaluation was performed following ($Z = \frac{(N_i - \bar{x}_i)}{\sigma_i}$) with $\bar{x}_i$ = plate mean of respective luminescence values and σ_i = S.D. of plate mean of luminescence values.

Reporter gene assays and mammalian two-hybrid analyses

Construction of pGLTfHRE wt and pGLTfHRE mut reporter plasmids carrying a hypoxia-responsive enhancer element derived from the human *Transferrin* gene was described previously[21]. Transfections for standard reporter gene experiments were carried out on 100 mm culture plates essentially as described earlier[17]. In brief, U2OS cells were co-transfected with 3 µg reporter plasmid or a mix of 1.5 µg reporter and 1.5 µg expression plasmids, respectively. Transfection efficiency was controlled by co-transfection of 20 ng pRL*SV40 renilla* luciferase reporter vector (Promega). RNA-interference (RNAi) mediated knockdown of HIF-1α, FIH-1 or p300 was achieved by transiently transfecting once (HIF-1α, p300) or twice (FIH-1) U2OS cells with 100 nM stealth RNAi duplexes (HIF-1α, 5'-caggacaguacaggaugcuugccaa-3'; FIH-1, 5'-gaaacauugagaagaugcuuggaga-3'; p300, 5'-ggauucgucugugauggcuguuuaa-3'; control, 5'-gcuccggagaacuaccagaguauua-3'; sense strands) using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Combined knockdown and reporter gene analyses were performed by sequentially transfecting cells with stealth RNAi duplexes 24 hours before subjecting them to PEI-mediated DNA transfection.

Mammalian two-hybrid analyses were performed using the mammalian Matchmaker system (Clontech) as described previously[14]. Expression vectors of HIF-1α NAD and CAD fused to Gal4 DBD were a kind gift of Dr. Sang (Drexel University, Philadelphia, U.S.A.)[22]. U2OS cells were transiently co-transfected with 1.5 µg of Gal4 DBD and 1.5 µg of VP16 AD fusion protein vectors together with 500 ng of *firefly* luciferase reporter vector pGRE5xE1b and 20 ng of pRL-SV40. Total transfected DNA amounts were equalized in each experiment using the corresponding empty vector. Luciferase reporter gene activities were determined using the dual-luciferase reporter assay system (Promega).

Fluorescence resonance energy transfer (FRET)

The full length ORF of human ETV4 was cloned into pENTR4 and subsequently recombined with pECFP-C1-DEST to obtain the expression vector for a cyan fluorescent ETV4 fusion protein[15]. U2OS cells were transiently transfected with the pECFP-ETV4 and pEYFP-HIF1 or pEYFP-HIF2 plasmids as recently described[59]. FRET was monitored under normoxic or hypoxic conditions (1% O_2 for 4 hours) 24-48 hours post-transfection.

Protein extraction and immunoblot analysis

Cells were washed twice and scraped into ice-cold phosphate-buffered saline. Soluble cellular protein was extracted with a high salt extraction buffer containing 0.4 M NaCl, 0.1% Nonidet P-40, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 1× protease inhibitor cocktail (Sigma). Protein concentrations were determined by the Bradford method and 50-80 µg of cellular protein were subjected to immunoblot analysis using the following antibodies: mouse monoclonal antibody (mAb) anti-human HIF-1α (clone 54/HIF-1α; BD Transduction Laboratories), mAb anti-ETV4 (PEA3(16); Santa Cruz Biotechnology) and rabbit anti-ETV4 (sdix20580002; Novus Biologicals), rabbit anti-human PHD2 (NB100-137; Novus Biologicals), mAb anti-FIH-1 (NBP1-30333; Novus Biologicals), mAb anti-p300 (554215; BD Pharmingen), and mAb anti-β-actin (clone AC-74; Sigma). Primary antibodies were detected with respective polyclonal anti-mouse or anti-rabbit sera conjugated to horseradish peroxidase (HRP; Pierce). Chemiluminescence signals were developed using Supersignal West Dura substrate

(Pierce) and images were acquired with a digital light imaging system (LAS 4000; Fuji).

mRNA quantification

Complementary DNA was generated by reverse transcription (RT) of 1-5 µg of total RNA using AffinityScript reverse transcriptase (Agilent). Transcript levels were determined by real-time quantitative (q) PCR using a SybrGreen qPCR reagent kit (Sigma) in combination with the MX3000P light cycler (Agilent). All RT-qPCR data are presented as ratios relative to ribosomal protein L28 mRNA values. Primer sets for human PHD2, CA9 and L28 have been described earlier[17,60].

Tissue microarray analysis

Clinico-pathological characterization and immunohistochemical analyses of selected components of the HIF-pathway utilizing a tissue microarray (TMA) consisting of 282 invasive breast cancer cases diagnosed at the Institute of Surgical Pathology (University Hospital, Zürich, Switzerland) have been described recently[13]. Sections of the same TMA were stained with a rabbit polyclonal anti-ETV4 antibody (HPA005768, Sigma) in a 1:100 dilution using an automated immunohistochemistry platform (Ventana BenchMark, Roche). An immunoreactive score (IRS) for ETV4 staining was calculated by multiplication of staining intensity (graded between 0 and 3) and the percentage of positive cells (graded between 0 and 4 with 0, nil; 1, <10%; 2, 10-50%; 3, 51-80%; 4, >80%) as quantified by a senior pathologist (G.K.). Nonparametric correlations between ETV4 expression and HIF-1/2α or HIF target genes were analyzed by calculating Spearman's rank correlation coefficient using PASW (IBM SPSS Statistics 18) software.

shRNA constructs and lentiviral infections

Expression vectors encoding shRNA sequences targeting human ETV4 and a non-coding control driven by the U6 promoter in a pLKO.1-puro plasmid were purchased from Sigma. Viral particles were produced in HEK293T cells using the ViraPower lentiviral expression system according to the manufacturer's instructions (Invitrogen). Infected PC3 cells were cultured in RPMI-1640 supplemented with 0.5 µg/ml puromycin.

Chromatin immunoprecipitation (ChIP)

ChIP assays from parental PC3 cells exposed to 20% or 0.2% O_2 for 4 and 24 hours were performed essentially as described previously[35]. The following antibodies were used for immunoprecipitation: rabbit anti-HIF-1α (ab2185; Abcam), and rabbit anti-ETV4 (sdix20580002; Novus Biologicals). Rabbit serum (011-000-001; Jackson ImmunoResearch) served as unspecific control. Enrichment of *PHD2* promoter chromatin was determined by PCR using the following oligonucleotides: *PHD2* forward 5'-gtatgccctgcgctcctc-3', reverse 5'-gctgagagaatagggcctgtg-3'.

Gene array analysis

Total RNA was extracted from pools of shRNA expressing PC3 clones with RNeasy (Qiagen). RNA integrity was evaluated using the Agilent 2100 Bioanalyzer. Genome-wide RNA levels were quantified using the human gene expression ShurePrint GE3 (8×60K) microarray according to the manufacturer's instructions (Agilent). All data were deposited in NCBI's Gene Expression Omnibus (GEO) and are publicly accessible through GEO accession number GSE32385 (http://www.ncbi.nlm.nih.gov/geo/).

Statistical analysis

If not otherwise indicated, results are presented as mean values ± standard error of the mean (S.E.M.) of at least three independent experiments. Column statistics applying paired *Student's* t-tests were calculated using GraphPad Prism version 4.0 (GraphPad Software).

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4 The ETS-transcription factor ETV4 is oxygen regulated and interacts indirectly with PHD2

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ABSTRACT

Oxygen homeostasis is a key mechanisms in cells and tissue that when disturbed eventually leads to the activation of the transcription factor family hypoxia inducible factors (HIF) which orchestrates the hypoxic response of the tissue by initiation of angiogenesis and erythropoiesis supporting gene products. The stability of HIF is oxygen-dependently regulated through HIF-prolyl-4-hydroxylase domain proteins (PHD). Since the two PHD genes, *PHD2* and *PHD3* are also inducible by HIF, a negative feedback loop is established. The ETS-transcription factor ETS translocation variant 4 (ETV4) was recently found to interact with HIFs to promote PHD2 transcription in hypoxia. Here we show that increased ETV4 levels positively correlate with PHD2 mRNA levels in various cancer cell lines. Furthermore, ETV4 potentially increase the hypoxic activation of promoters that contain a distinct sequence architecture surrounding the HIF-binding site (HBS). Hence, next to *PHD2* promoter activity other promoters such as *CA9* are shown to have an increased activity in the presence of ETV4. Additionally, ETV4 protein is more abundant in hypoxia, in PHD2 knockdown cells, and in cells treated with a PHD-inhibitor. Mammalian-2-hybrid experiments suggest a possible interaction between ETV4 and PHD2.

4.1 Introduction

Recently, we reported a new level of interplay between the ETS- and HIF-pathways in cellular adaptation to low oxygen environments[1]. A member of the large ETS-transcription factor family, ETS variant 4 (ETV4, also known as PEA3 or E1AF), was found to contribute to hypoxic *PHD2* gene expression[1]. ETV4 was demonstrated to affect not only *PHD2* expression, but also the *transferrin* regulatory region in an HIF-DNA-binding site dependent manner. Similarly, we observed an increase in hypoxic *PHD2* promoter activity with the overexpression of the structurally similar family members ETV5, but not ETV1. Further, we showed that CAIX and PHD3 expression are highly ETV4-dependent, too. The question remains why certain hypoxia-induced genes are super-induced by ETV4 whereas others remain unaffected. It is widely accepted that the HBS adjacent regions are of high importance for the full functional power of HIF[2,3]. This was equally shown to be true for the HBS located in the *PHD2* promoter (cf. 2.2.3 et seqq.). The consensus binding sequence of the ETV4 family members was defined as 5'-$^{A}/_{C}$GGAAGT-3' and is located in different promoters of pro-metastatic matrix metalloproteases (MMPs), like MMP-1 and MMP-9 as well as human stromelysin-1, matrilysin, collagenases type I and IV, leading to tissue rearrangement - a common event in metastatic processes[4–11]. A link between ETS-transcription factors and the HIF-pathway was described previously[1,12–14]. In line with the observation promoter analysis revealed putative ETS binding sites in HIF-2α dependent genes that were located in 90% of the cases even in proximity to the putative HREs[15]. However, the specific common binding motif of the ETV4 family members is not found in the neighboring sequence of the PHD2 HBS. ETV4, ETV5 and ETV1 have been shown to be involved in the development of branching tissue as well as the migration and invasion of various cancers[16–22]. The ETVs are degraded via the ubiquitin-proteasomal pathway. When the degradation pathway is disturbed an accumulation of ETVs leads to the expression of gene products (MMPs etc.) that support the invasive features of cancer cells, i.e. prostate cancer[23–25]. For example, recent reports showed that deficiency in constitutive photomorphogenesis protein 1 homolog (COP1), an E3-ubiquitin ligase, results in the accumulation of ETVs and produces increased cell proliferation, hyperplasia, and early prostate intraepithelial neoplasia being a pre-stage of prostate adenocarcinomas[23,24].

We aimed for the investigation of the interaction between HIF and ETV4 signalling. We report that ETV4 is intimately involved in the HIF-pathway by a positive regulation in hypoxia. Here, we hypothesize an interaction with PHD2. The data suggests that the transcription factor ETV4 is similar in hypoxic activation and stability to the HIF-α subunits and needs to be further analyzed to understand its role in hypoxia-mediated transcription.

4.2 Results

4.2.1 High ETV4 mRNA levels correlate with high PHD2 mRNA levels in various cancer cell lines

The ETS-transcription factor ETV4 was recently shown to enhance *PHD2* promoter activity by co-activation of the hypoxia inducible factors (HIFs), suggesting that the oncogene ETV4 plays a role in solid tumor progression of hypoxic cancers[1]. We hence tested a range of different human cancer cell lines reported to express ETV4, such as human fibrosarcoma (HT1080), osteosarcoma (U2OS), hepatocellular liver carcinoma (HepG2), colorectal cancer (HCT116) and breast cancer (MDA-MB-231), for their ETV4 and PHD2 mRNA levels in normoxic and hypoxic conditions. As reported previously, ETV4 mRNA levels remain unchanged following hypoxic treatment of the cells, whereas PHD2 levels are induced in hypoxia. In line with the observation that ETV4 is super-inducing the *PHD2* promoter, PHD2 mRNA levels are highest in cell lines that most strongly express ETV4 (HepG2 and U2OS) (fig. 1). This suggests that ETV4 and PHD2 expression levels positively correlate in various cancer types and that ETV4 expression might influence the hypoxic phenotype of a cancer.

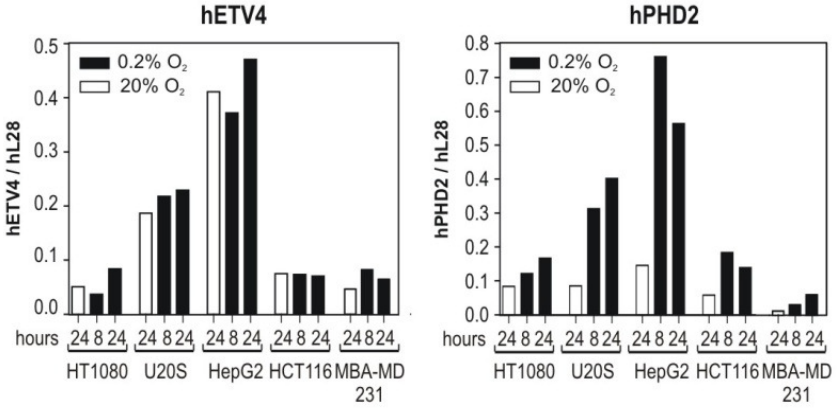

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Figure 1. ETV4 and PHD2 mRNA levels positively correlate with each other in various human cancer cell lines.

The indicated human cancer cell lines reported to express ETV4 were tested for their ETV4 and PHD2 mRNA levels in normoxic (24 h) and hypoxic conditions. Total RNA was extracted 8 h and 24 h respectively after hypoxic incubation and mRNA levels of ETV4, PHD2 and L28 were quantified by reverse transcription qPCR.

4.2.2 The transcription factor ETV4 shows strong, hypoxically inducible activation of those HBSs that are structurally similar in 5'- and 3'- architecture

ETV4 not only superinduces the activity of the *PHD2* promoter, but also of the *transferrin* promoter in a HIF-dependent manner[1]. Therefore, we compared 107 known HIF target genes, all of them sharing the consensus sequence 5'-RCGTG-3', for similarities in the 5'- and 3'- sequences surrounding the HBS (fig. 2A). Interestingly, the 5'- and 3'- area of the *PHD2* promoter HBS seems to be similar to the *transferrin* promoter, indicating a structural relationship needed for an ETV4-HIF cooperation to increase the activity of the HBS. This conservation - especially of the 5'-region - was found in several further HBSs. We therefore transfected the corresponding reporter gene constructs together with ETV4. ETV4 mostly enhanced the reporter activity, when a structural similarity of the 5'-region existed, such as the HBS of *carbonic anhydrase IX* (*CA9*) (fig. 2B). This finding is in line with our previous report where we could demonstrate a strong ETV4-dependency of the *CA9* locus[1]. When the HBS-flanking regions were different, e.g. in the *lysyl oxidase* (*LOX*) and *erythropoietin* (*EPO*) HBSs, no superinduction was achieved. Thus, the specificity of those target genes that are superinduced through the collaboration of ETV4 and HIF might be determined by the expanded consensus sequence 5'- *GTGT*ACGTG*CA*-3'.

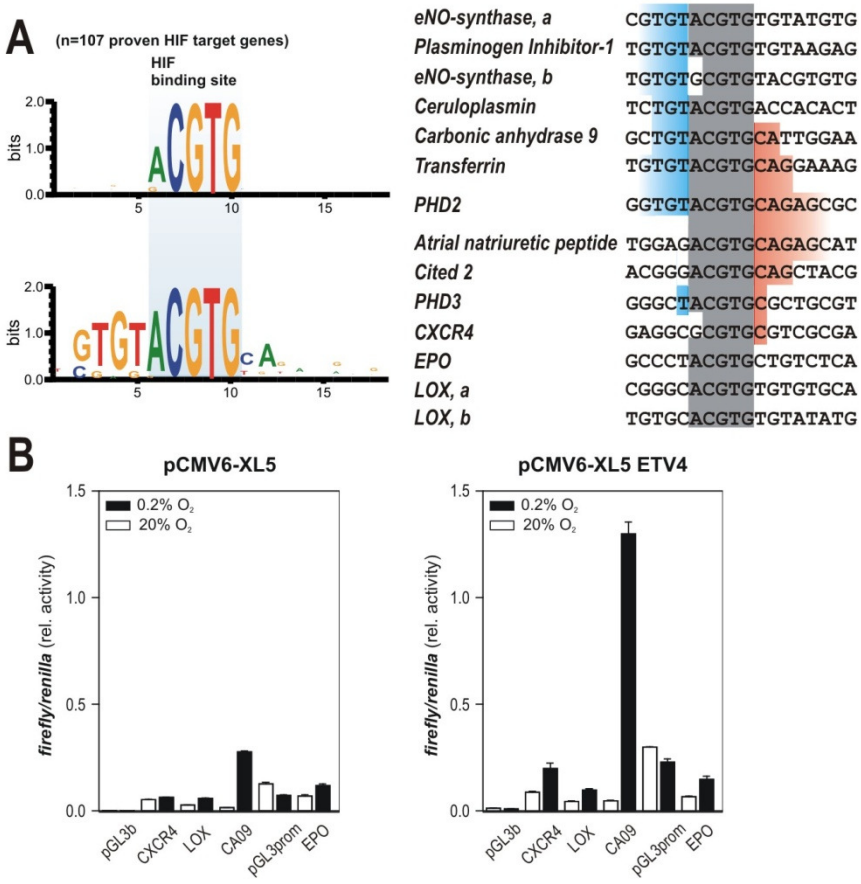

Figure 2. ETV4 enhances the transcriptional activity of HBS that show a similar *PHD2* HBS surrounding architecture.

(A) The upper panel shows an overview of HBSs of 107 validated HIF-1α target genes that were examined for the commonly occurring motif 5'-RCGTG-3' using the Gibbs Motif Sampler of CisGenome. The lower panel represents only sequences similar to the HBS in the *PHD2* promoter region. Therefore, the other HBS-regions were compared to the *PHD2* HBS which served as a standard template. The height of each letter is proportionate to its occurrence frequency, with the most frequent at the top. The height of each stack is then normalized to reflect the information content of the sequence at the respective position (measured in bits). Interestingly, the 5'-region and to the same extent the 3'-region surrounding the HBS seem to be conserved, too, extending the consensus sequence to 5'-*GTGT*RCGTG*CA*-3'. A selection of HBS motifs is shown on the upper right panel highlighting those positions of nucleotides that are conserved. **(B)** Luciferase reporter gene assay of 4 different HBS-containing regions: *C-X-C chemokine receptor type 4* (*CXCR4*), *lysyl oxidase* (*LOX*), *carbonic anhydrase 9* (*CA9*), *erythropoietin* (*EPO*) and their empty vector controls. Following co-transfection without (left) or with (right) ETV4 expression constructs in U2OS cells, for 24 h cells were incubated overnight under 20% O_2 (normoxia) or 0.2% O_2 (hypoxia). ETV4 could enhance the reporter activity of the *CA9* promoter, but none of the other promoters, suggesting that ETV4 specifically superinduces transcriptional activity of certain HBSs similar to the *PHD2* promoter. Bars represent means ± S.E.M. of 3 independent experiments.

4.2.3 Stable knock down of ETV4 in U2OS cells does not affect hypoxia-induced HIF target genes

In order to analyze the effects of ETV4-depletion in U2OS cells we established a stable shRNA-mediated ETV4-knockdown pool (fig. 3A). By testing several target genes that were reported in literature to be dependent on ETV4 expression (*cyclooxygenase2* (*COX2*)[26,27], *MMPs* (*MMP-2*)[5,6], *osteopontin* (*OPN*)[28], *plasminogen activator-urokinase* (*PLAU*)[29,30] or hypoxia-induced (*PHD2*[31], *PHD3*[32], *vascular endothelial growth factor a*[33] (*VEGFa*)) we aimed at elucidating if ETV4 silencing also affects the hypoxic response. Some of these genes have been reported to be both, ETV4 target genes and simultaneously involved in the cellular response to hypoxia such as COX2[30,34] and VEGF[35,33,36]. ETV4 target genes like *COX2*, *MMP-2*, *OPN* and *PLAU* were downregulated in these cells, whereas other genes remained unaffected. However, some genes such as *PHD2* as well as other proven hypoxia-induced target genes (i.e. PHD3, VEGFa, transferrin receptor) did not reveal any ETV4-dependency, although genome-wide microarray data from PC3 cells suggested so[1].

U2OS cells stably knocked down for ETV4 were transfected with the *PHD2* promoter region (P2P (-607/+3)) and exposed to hypoxia. The knockdown of ETV4 did not differ from wildtype cells with respect to hypoxic inducibility of the reporter construct (fig. 3B). A possible explanation for this unexpected finding is that ETV4 is most probably not sufficiently expressed in U2OS cells since ETV4 protein levels were close to their detection limit. Given that ETV4 is an oncogene driving tumor development when overexpressed, the knockdown in a cell line that does not express high levels of this protein appears to be the wrong approach. Therefore, ETV4 silencing should be applied rather in a cancer cell line that naturally expresses more ETV4.

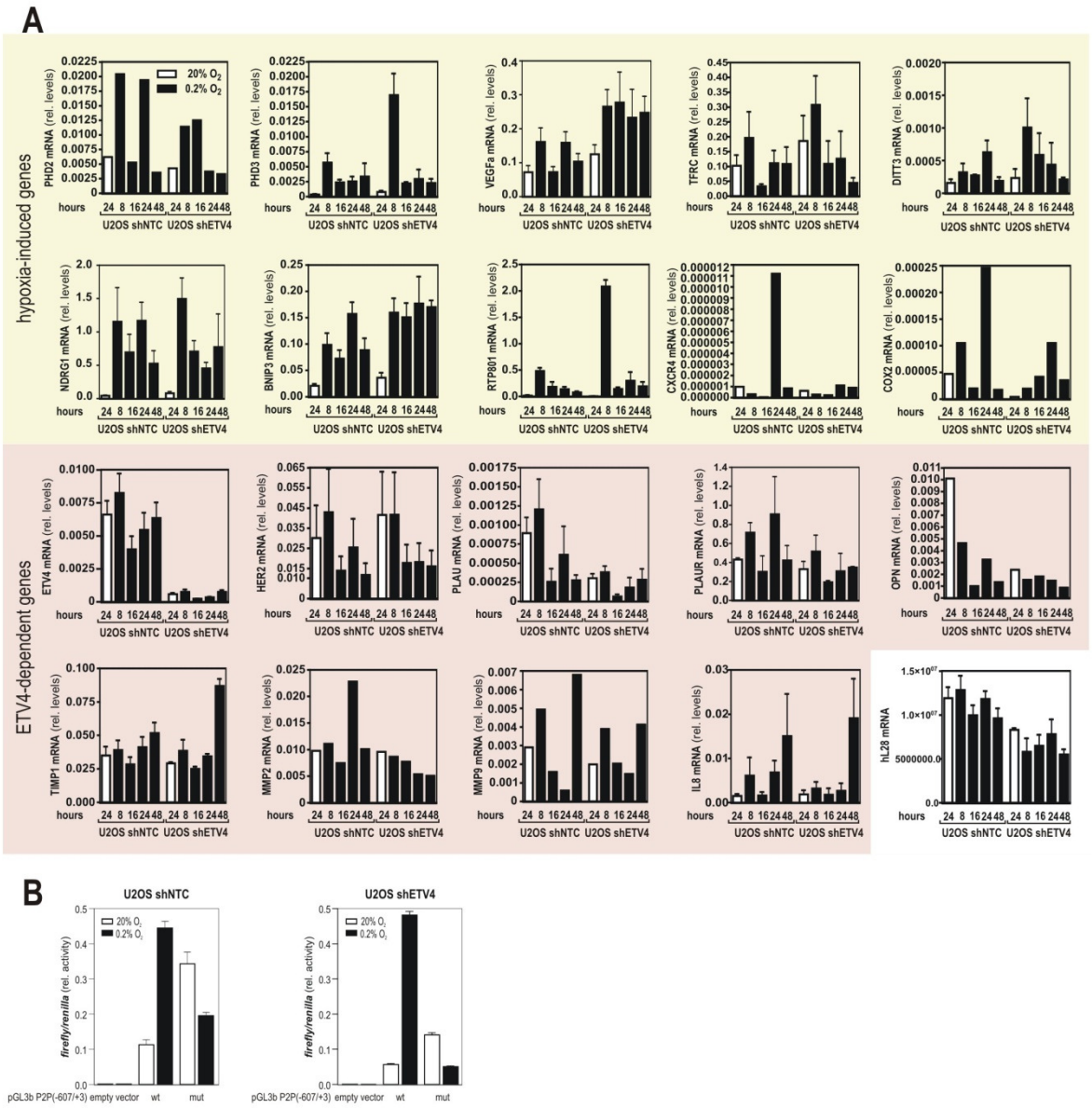

Figure 3. Role of ETV4 in the regulation of established ETV4 and HIF target genes *in vitro*.

(A) In a stable pool of ETV4-knockdown U2OS cells, mRNA levels of a panel of in literature primarily reported HIF- (yellow background; PHD2[37], PHD3[32], VEGFa[38], TFRC[39], NDRG1[40], BNIP3[41], RTP801[42], CXCR4[43], DITT3, COX2[34]) and ETV4-dependent (pink background; Her2[44], PLAU[30], PLAUR[45], OPN[28], MMP2 and MMP9[46], IL8[47]) target genes which either show tendency to require ETV4 for efficient induction (COX2, RTP801) or that remain unaffected by the ETV4 knock-down (NDRG1 etc.) were mRNA was quantified by RT-qPCR. Measured from three independent experiments except for Cox-2, MMP-2, MMP-9, OPN, CXCR4 and PHD2 that were determined from a single experiment. **(B)** Stable shETV4 knockdown U2OS clones were transiently transfected with the *PHD2* promoter driven reporter gene plasmid *pGL3 P2P(-607/+3)* in its HBS wildtype or mutant form. 24 h after transfection, cells were incubated overnight under 20% O_2 (normoxia) or 0.2% O_2 (hypoxia). Mutation of the HBS leads to the abrogation of hypoxic inducibility. Bars represent means ± S.E.M. for 3 independent experiments.

4.2.4 ETV4 protein is increased in hypoxia

Since ETV4 overexpression was demonstrated to induce PHD2 expression levels in hypoxia[1], we examined if a transient silencing rather than a stable ETV4-downregulation in U2OS cells would affect PHD2 expression (cf. 4.2.3). Therefore, we carried out a transient siRNA-mediated knockdown of ETV4. We expected to decrease hypoxia-induced PHD2 expression since ETV4 as a HIF co-activator is missing. As observed for the stable ETV4 knockdown, the transient silencing equally did not change PHD2 mRNA or protein expression levels in U2OS cells underlining the importance of an adequate cell model with sufficient ETV4 expression (fig. 4A and B). While ETV4 mRNA levels remained unaffected by hypoxia (fig. 4A), excluding ETV4 as HIF target gene, ETV4 protein levels significantly increased (fig. 4B, quantification). This observation suggests that hypoxia regulates ETV4 protein. The negative feedback loop of HIF-α and PHD2/PHD3 is most prominent and balances the cellular hypoxic response. When this negative regulation process is disturbed by a silencing of the main cellular oxygen sensor PHD2, HIF-α subunits accumulate in normoxia. As ETV4 protein levels seem to be similarly regulated by hypoxia as HIF-α subunits we performed a transient PHD2 knockdown. Interestingly, a transient silencing of PHD2 resulted in increased ETV4 protein levels in normoxic conditions similar to HIF-1α (fig. 4B). This could hint to a hydroxylation-dependent regulation of ETV4 through PHDs that is inhibited when the O_2-concentration is low or when PHDs are silenced.

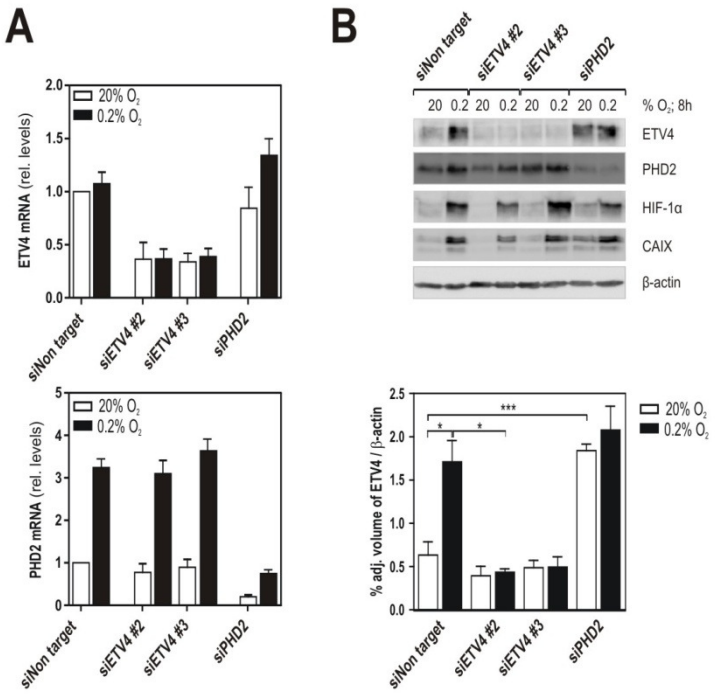

Figure 4. ETV4 protein is increased in hypoxia and shows high levels in normoxic conditions when PHD2 is knocked-down.

(A and B) Transient knockdown by siRNA of ETV4 or PHD2, respectively. U2OS cells were cultured under normoxic (20% O_2) or hypoxic (0.2% O_2) conditions for 8 h. **(A)** Total RNA was extracted after 8 h of normoxic or hypoxic incubation, and mRNA levels of ETV4, PHD2 and ribosomal protein L28 were quantified by RT-qPCR. **(B)** Cellular proteins were extracted and endogenous ETV4, PHD2, HIF-1α, CAIX and β-actin levels were analyzed by immunoblotting. The lower graph represents the immunoblot quantification of ETV4 of 4 independent experiments. *P*-values were obtained by unpaired Student *t*-tests (**P*<0.05, ****P*<0.001).

4.2.5 ETV4 shows rapid protein accumulation when treated with DMOG or with proteasomal inhibition by MG132

In order to confirm a possible hydroxylation-dependent regulation of ETV4 we applied in a time dependent manner the PHD-inhibitor dimethyloxalylglycine (DMOG) to U2OS cells and analyzed ETV4 protein levels. We could demonstrate that like hypoxia, the PHD inhibitor DMOG also induced ETV4 protein levels in U2OS cells (fig. 5A). ETV4 protein levels peaked already after one hour, whereas HIF-1α induction started only after 2 hours of treatment. As a conclusion the observed ETV4 protein accumulation in hypoxia is not HIF-dependent since ETV4 predates HIF-1α stabilization.

Furthermore, both ETV4 and HIF were induced by proteasomal inhibitor MG132 (fig. 5B). Interestingly, also here ETV4 induction preceded HIF-1α induction. ETV4 and HIF-1α accumulated after 30 min and 4 hours, respectively. Consequently, this result hints at a shorter half-life of the ETV4 protein as compared to HIF-1α.

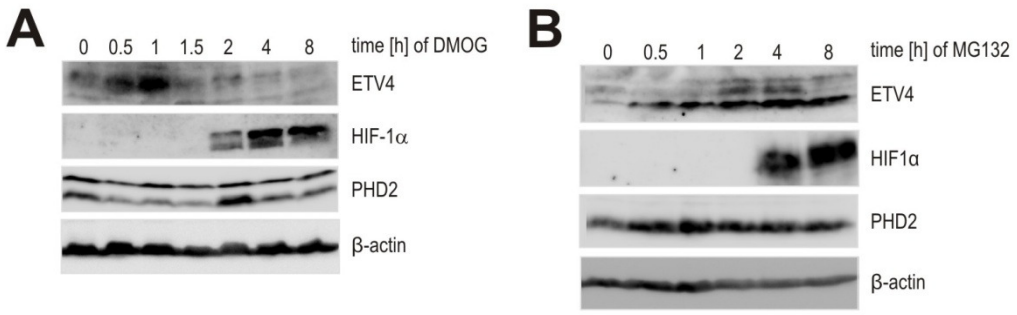

Figure 5. ETV4 shows rapid protein accumulation when U2OS cells were treated with the PHD-inhibitor DMOG or proteasomal inhibition by MG132.

(A and B) Immunoblot detection of ETV4, PHD2, HIF-1α and β-actin. U2OS cells were incubated for the indicated time in the presence of solvent control, 2 mM DMOG **(A)** or 5 μM MG132 **(B)**.

4.2.6 Interaction of ETV4 with PHD2 under hypoxic conditions?

To assess the possible interaction between ETV4 and PHD2, we carried out fluorescence resonance energy transfer (FRET) analysis as well as mammalian-2-hybrid assays. The FRET data showed no interaction between ETV4 and PHD2 under normoxic conditions (fig. 6A and B). This finding could be supported in the mammalian-2-hybrid approach, where the two proteins are coupled either to a VP-16 activation domain (AD) or to a GAL-4 DNA-binding domain (DBD), respectively. If the two proteins interacted, they would activate the GAL4 response element-driven firefly luciferase reporter construct. However, under hypoxic conditions a weak interaction could be detected between PHD2 and ETV4 in a HIF-1α dependent manner (fig. 6C and D).

A possible explanation could be that ETV4 is a hydroxylation target of PHD2. Sequence analysis of the ETV4 revealed a large number of proline residues that could potentially be hydroxylated by PHDs. However, the known HIF-1α hydroxylation sequence site LxxLAP was not found in the ETV4 protein, but two close, although not proven to be functional sites - LxxLxAP- were found in the C-terminal end of the ETV4 protein (fig. 6E).

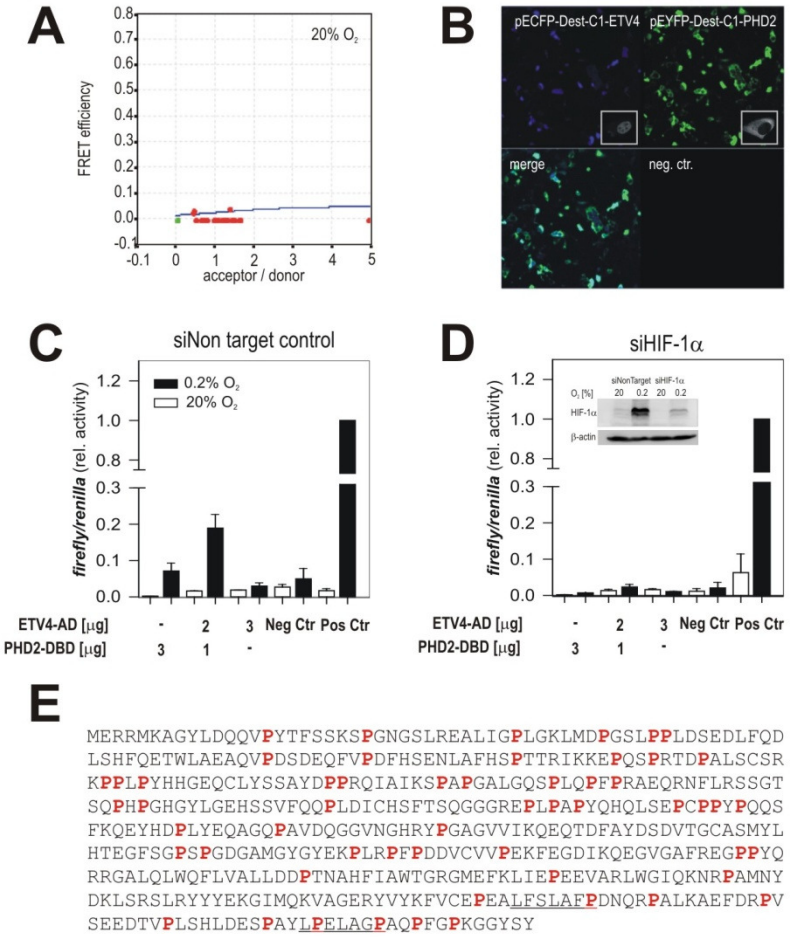

Figure 6. ETV4 does not interact in normoxia with PHD2 in FRET analysis. ETV4-PHD2 interaction is regulated by oxygen.

(A) U2OS cells were transiently transfected with the indicated CFP or YFP plasmids, and FRET analysis was performed at 20% O_2 or 1% O_2 24 h post transfection. FRET efficiencies for CFP-ETV4 and YFP-PHD2 fusion protein pairs were calculated from 20 - 40 randomly selected cells which displayed various fluorescent acceptor/donor ratios. Scatter plots were fit to a single-site binding model. FRET efficiencies are given as the percentage of transferred energy relative to the energy absorbed by the donor. The FRET signal (red dots) is below the negative control curve of none-interacting proteins (blue). **(B)** Microscopic images showing the subcellular localization of the exogenous proteins. Inlay shows single cell expression of pECFP-Dest-C1-ETV4 and pEYFP-Dest-C1-PHD2. **(C and D)** Transient knockdown U2OS cells were generated for non target control **(C)** or HIF-1α **(D)** via siRNA. Subsequently, those cells were transiently transfected with GAL4-DBD and VP16-AD fusion protein vectors and a GAL4-response element-driven firefly luciferase reporter gene, as well as a renilla luciferase control vector. The interaction between AD-HIF2α ODD and DBD-PHD2 was used as positive control and AD-CP1 / DBD-p53 functioned as negative control. Following transfection, the cells were incubated under normoxic (20% O_2) or hypoxic (0.2% O_2) conditions, and luciferase reporter gene activities were determined 24 h later. Firefly / renilla luciferase activity ratios were normalized to the normoxic positive control DBD-PHD2 /HIF-2α ODD-AD co-transfection, which was arbitrarily defined as 1. **(Inlay)** To confirm siRNA-efficiency HIF-1α and β-actin levels were

analyzed by immunoblotting. **(E)** The ETV4 protein sequence contains a multitude of proline residues (bold red). The prolyl-hydroxylation consensus sequence site LxxLAP found in the HIF-1α subunit is not incorporated in the ETV4 protein sequence. However, two putative sequences, although not absolutely identical to those motifs described in HIF-1α, are found in the C-terminus of the protein (LxxLAxP; underscored).

4.3 Discussion

In this study, we compared the ETV4-transcription factor pathway with the hypoxic PHDs-HIF-1α pathways in human cancer cells, such as U2OS, PC3 and HepG2. Hypoxia is known to regulate the stability and activity of the HIF-α subunits and thus target gene expression. Our data propose that the mRNA levels of ETV4 and PHD2 positively correlate in various cancer cell lines suggesting that the degree of ETV4 expression might have an influence on the hypoxic phenotype and malignantly on a cancer. However, for a broader and statistically relevant correlation it would be of interest to enlarge this analysis by including a bigger range of cancer cell lines and primary cells. ETV4 family members share a high degree of sequence similarity. The ETS-domain, responsible for the DNA-interaction shares 95% of similarity and the transactivation domains still roughly 85%. Recently, we have reported that ETV4 is able to increase the transcriptional activity of the *PHD2* promoter as well as of a *transferrin* reporter construct in a HBS-dependent manner[1]. Although we showed that ETV5 is also capable to compensate for ETV4 action, ETV4 has more physiological relevance since in various tissues it is more widely expressed than ETV5. Functional redundancy might explain why neither transient RNAi-mediated nor lentiviral-mediated stable knockdown of ETV4 affected PHD2 expression levels or *PHD2* promoter activity in U2OS cells. However, most probably the osteosarcoma cell line U2OS is a mediocre cell model for investigating the effects of PHD2 expression, since ETV4 mRNA and protein expression levels are not sufficiently abundant in those cells.

In this study, we report that the reason of an ETV4-mediated super-induction of specific hypoxia-induced target genes maybe lie in the common architecture of the 5'- and 3'-regions adjacent to the HBS. This structure seems to be required for the increased trans-activation by ETV4. This hypothesis is underlined by the observation that the *carbonic anhydrase 9* (*CA9*) promoter - that is similar to the PHD2 HBS region - also was showing a super-induction under hypoxic conditions in the presence of ETV4. In contrast, when the HBS-flanking region is dissimilar to the PHD2 HBS, the super-induction does not take place. This observation would argue for the synergistic interplay between ETV4 and HIF when distinct sequence

architectures are given. A highly informative and unbiased approach to support this hypothesis would be the genome-wide measurements of protein-DNA interaction by chromatin immunoprecipitation (ChIP) followed by quantitative measurements and high-throughput sequencing of the transcriptome. These combined techniques could be used to determine the common features of hypoxia-induced and ETV4-dependent target genes. However, the interaction of the ETS-DNA-binding domain with the DNA appears rather unlikely in this combination, since despite the strong conservation of ETV4 family members in their DNA-binding domain (5% of dissimilarity), ETV1 did not super-induce the *PHD2* or *transferrin* promoter. Additionally, the characteristic consensus sequence 5'-$^{A}/_{C}$GGAAGT-3' is not found in HBS-adjacent regions. We therefore propose a model in which ETV4/5 adopt a particular conformation by binding to p300/CBP and HIF. This conformation supports the interaction of other ETV4/5 domains (absent or different in the ETV1 protein) to interact with the HBS-adjacent DNA-regions to super-induce specific hypoxia-induced target genes.

DNA binding and co-activatory features of ETV4 in hypoxic gene expression might be additionally linked to the finding that ETV4 is regulated in U2OS cells by hypoxia. This would lead to the discovery of a new oxygen-dependent transcription factor and implies physiological importance of the hypoxic super-induction of distinct genes in special cell types. Strikingly, ETV4, as well as HIF-1α shows an accumulation of protein under hypoxia, through the treatment with the PHD-inhibitor DMOG or proteasomal inhibition by MG132[48]. The very high turnover of ETV4 could speak for the high transcriptional power of ETV4 and the importance to eliminate this (co-) transcription factor from the cell when not needed. Similarly the HIF-α subunits are strictly regulated and lead, when unregulated, to cancer development. PHDs regulate the stability of HIF-α subunits through the hydroxylation of distinct proline residues[49–53]. The ETV4 subfamily, similar to HIF-1α, also undergoes posttranslational modification as phosphorylation or sumoylation and might also include hydroxylation[23,25,54–67]. An indication could be the multiple bands of ETV4 in SDS-PAGEs. Additionally, PHDs were reported to hydroxylate other proteins[68]. We suggest that the hypoxic regulation takes place on a posttranslational level possibly through the prolyl hydroxylation of ETV4 by PHDs. This is evidenced by the accumulation of ETV4 under hypoxia, through the treatment with the PHD-inhibitor DMOG or the transient silencing of PHD2, although regulatory mechanisms based on

translational levels still need to be examined. It is conceivable that, as the HIF-α subunit is no longer ubiquitinylated by the von-Hippel-Lindau ubiquitin ligase, COP1 is no longer able to mark ETVs under hypoxia for degradation. No hypoxic regulation of ETV4 is observed In PC3 cells[1]. Generally, PC3 cells express high levels of ETVs due to the deficiency in the E3-ubiquitin ligase COP1 that leads to constant ETV accumulation. We suggest that in COP1-expressing cells the hydroxylation of ETV4 prolyl residues by PHDs inhibit the interaction between Cop1 and ETV4. As a result this leads to an accumulation of ETV4 protein. In PC3 cells the degradation process is impaired and it triggers an oxygen-independent accumulation of ETV4. Especially the increased normoxic ETV4 protein levels in PHD2 silenced cells hint to the conjecture that PHD2 is possibly involved in the regulation of ETV4 degradation. It would be of further interest if also the knockdowns of PHD1/3 have the same effects on ETV4 protein levels. That PHDs are capable to hydroxylate other target proteins than the HIF-α subunit was indirectly shown with several other proteins, such as RbpI, the large subunit of RNA polymerase II, activating transcription factor-4 (ATF4) or pyruvate kinase M2 (PKM2)[68–71]. However, we did not pursue if hydroxylated prolines are detectable in ETV4 by mass spectroscopy, since the proline-rich nature of the protein is hindering the analysis[72].

Since PHD2 and PHD3 are HIF target genes on their own, their induction in hypoxia partially compensates for the decreased enzymatic activity in oxygen-deprivation by an increased amount of proteins and consequently recovers basal proteasomal degradation of HIF-1α[73]. ETV4 downregulation decreased *PHD3* mRNA induction in hypoxia, suggesting that ETV4 is also a positive transcriptional regulator of PHD3 expression and might thereby indirectly influence the degradation of HIF-1α[1]. Furthermore, our results show that ETV4 and HIF-1α exhibit a similar response to modulators of PHD activity, but that they are differently fine-tuned during the hypoxia-mimicking DMOG time course. ETV4 levels are high in very early hypoxia, whereas HIF-1α is induced later during acute hypoxia, but both factors are degraded in prolonged hypoxia-mimicking conditions. This proposes that HIF and ETV4 have different roles in the hypoxic cellular response, underlined by the tendency of ETV4 to super-activate especially those target genes that are responsible in both, acute and chronic hypoxia[74].

In summary, we could show that the ETV4 and hypoxia pathways seemed to be interlinked at the level of PHD2/3 expression regulation and protein-protein interactions[1]. ETV4 protein was demonstrated to be more abundant in hypoxia, in PHD2 knockdown cells, and in cells treated with a PHD-inhibitor. Additionally, a possible interaction between ETV4 and PHD2 is suggested by mammalian-2-hybrid experiments. The common feature for the synergistic interplay of ETV4 and HIF might be a specific sequence architecture surrounding the HBS.

4.4 Materials and methods

Plasmid constructs

PHD2 promoter (P2P) containing the wildtype and mutated HBS in pGL3basic luciferase vector were obtained from DP Stiehl[37]. These constructs were modified employing a 5'- truncation and a 3'- start codon fusion to the luciferase open reading frame by using standard restriction-mediated cloning techniques[1]. The *PHD2* luciferase reporter plasmid contained *PHD2* promoter sequences extending from –607 to +3(ATG start codon) (transcriptional start site unknown) and has been published previously[75] Cloning of pM-PHD2-Gal4 DNA binding domain (DBD) and ETV4-VP16 activation domain (AD) expression vector (pcDNA3.1) plasmids was carried out using Gateway technology (Invitrogen, Basel, Switzerland) as described previously[76]. The ETV4 family member plasmids pSV-ETV1, pSV-PEA3 (ETV4) and pSV-ERM (ETV5) were kindly provided by J-L Baert (France)[59]. The human *CXCR4* promoter and the intronic region cloned into pGL2b (Promega) was a kind gift of W Krek[43]. Erythropoietin (EPO) 3' hypoxia response element was kindly provided by RH Wenger[77].

Protein extraction and immunoblot analysis

Cells were washed twice and scraped into ice-cold phosphate-buffered saline. Soluble cellular protein was extracted with a high salt extraction buffer containing 0.1% Nonidet P-40 essentially as described before[78]. Protein concentrations were determined by Bradford method using bovine serum albumin as a standard, and 50 – 80 µg of protein were subjected to immunoblot analysis. According to standard procedures using PVDF membranes (Roth)[76]. The following antibodies were used: mouse anti-human HIF-1α (Transduction Laboratories BD Biosciences), rabbit anti-human PHD2 (Novus Biologicals), mouse anti-β-actin (Sigma), mouse anti-ETV4 (Santa Cruz Biotechnology) and mAb anti-CA09 (M75, kindly provided by S. Pastorekova), were detected with secondary polyclonal goat anti-mouse and anti-rabbit antibodies coupled to horseradish peroxidase (HRP) (Pierce). Chemiluminescence detection was done using Supersignal West Dura (Pierce) and quantified with a CCD camera based light imaging system (Fuji, LAS 4000) followed by quantification with Quantity One software (Bio-Rad).

Cell culture and transfections

Human human fibrosarcoma cell line (HT1080), osteosarcoma cell line (U2OS), hepatocellular liver carcinoma cell line (HepG2), colorectal cancer cells (HCT116) and breast cancer cell line (MDA-MB-231) were grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum (FCS) and antibiotics (penicillin 50 IU/ml and streptomycin 100 µg/ml, Invitrogen) (Gibco-BRL) as described previously[79]. Hypoxic conditions were generated by incubation of cells in an InvivO2 400 hypoxic workstation (Ruskin Technologies). The oxygen concentration was maintained at 0.2-1%, with the residual gas being 94-94.8% nitrogen, and 5% carbon dioxide. Transfections were performed using polyethylenimine (Polysciences) as described previously[73].

Dual-luciferase assay

Cells were cotransfected with 20 ng pRLSV40 renilla-luciferase reporter vector (Promega) and 3 µg reporter plasmid or 1.5 µg reporter and additionally 1.5 µg expression plasmid, respectively. After 24 hours of normoxic and further 24 h of hypoxic (0.2% O_2) incubation cells were lysed in 15 µl of passive lysis buffer (Promega). Luminescence was immediately analyzed with a microplate luminometer (Berthold) using dual-luciferase reporter assay system (Promega).

Mammalian two-hybrid analyses were performed using the mammalian Matchmaker system (Clontech) as described previously[76]. U2OS cells were transiently co-transfected with 1.5 µg of Gal4 DBD and 1.5 µg of VP16 AD fusion protein vectors together with 500 ng of firefly luciferase reporter vector pGRE5xE1b and 20 ng of pRL-SV40. Total transfected DNA amounts were equalized in each experiment using the corresponding empty vector. Luciferase reporter gene activities were determined using the dual-luciferase reporter assay system (Promega).

Fluorescence resonance energy transfer (FRET)

The full length open reading frame of human ETV4 was cloned into pENTR4 and subsequently recombined with pECFP-C1- DEST to obtain the expression vector for a cyan fluorescent ETV4 fusion protein[80]. U2OS cells were transiently transfected with the pECFP-ETV4 and pEYFPHIF1 plasmids as recently described (22), and

FRET was monitored under normoxic or hypoxic conditions (1% O_2 for 4 hours) 48 hours post transfection.

mRNA quantification

Total RNA was obtained as described previously[76]. Complementary strand DNA synthesis was performed with 1–5 µg total RNA using reverse transcriptase (AffinityScript, Stratagene). Complementary DNA / mRNA levels were determined by reverse-transcription (RT) quantitative (q) -PCR using SybrGreen qPCR reagent kit (Sigma) and the MX3000P light cycler (Stratagene). Ribosomal protein L28 mRNA was assessed to equal input levels. All qPCR data are presented as ratios relative to L28 values. Primers were as follows: hL28 forward, 5'-gcaattccttccgctacaac-3'; hL28 reverse, 5'-tgttcttgcggatcatgtgt-3'; hPHD2 forward, 5'-gaaagccatggttgcttgtt-3'; hPHD2 reverse, 5'-ttgccttctggaaaaattcg-3'; hETV4 forward, 5'-acggacttcgcctacgactca-3'; hETV4 reverse, 5'-cctggcgacctcctcaggct-3'.

RNA interference

U2OS cells were transfected with 100 nM siRNA duplex oligonucleotides using Lipofectamine 2000 (Invitrogen). The following RNA interference sequences (Invitrogen) were used:

control siRNA, forward 5′-gcuccggagaacuaccagaguauua-3′;

ETV4_oligo 2 siRNA, forward 5′-ggguggugaucaaacaggaacagac-3′;

ETV4_oligo 3 siRNA, forward 5′-ugacaucugagucguaggcgaaguc-3′;

PHD2 siRNA, forward 5'-uaacaagcaaccauggcuuucgucc-3'.

shRNA constructs and lentiviral infections

Expression vectors encoding shRNA sequences targeting human PHD2 and ETV4 driven by the U6 promoter in a pLKO.1-puro plasmid were purchased from Sigma. Viral particles were produced in HEK293T cells using the ViraPower lentiviral expression system according to the manufacturer's instructions (Invitrogen).

Statistical analysis

If not differently indicated, results are presented as mean values ± standard error of the mean (S.E.M.) of at least three independent experiments. *P*-values were obtained by unpaired *t*-tests (**P*<0.05, ***P*<0.01, ****P*<0.001).

4.5 References

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5 Discussion

5.1 The key players in hypoxia

An imbalance between oxygen delivery and consumption leads to a restricted oxygenation of tissues. Such an undersupply of oxygen is involved in a variety of pathologies like ischemia, stroke, inflammation and cancer. The unique responsiveness to altered oxygen environments together with the striking conservation of the PHD-HIF-system in metazoans indicates the central role of HIFs - literally the *hypoxia-inducible* transcription factors[1].

As a result of the negative HIF-PHD2/3 feedback loop we consider the transcriptionally hypoxic regulation of the *PHD2* gene as important since the PHD2 enzyme abundance influence its enzymatic capacity[2,3]. For a profound understanding of the transcriptional regulation we thoroughly studied the *PHD2* promoter architecture and aimed to find indications on further regulatory mechanisms of its gene expression.

To date, HIF is the only known transcription factor influencing the *PHD2* gene transcription. Since eukaryotic gene expression is a multi-step process requiring the complex transcription machinery to interact with promoter DNA and initiate transcription, it is not surprising that several studies have identified other nuclear regulators that contribute to the full spectrum of transcriptional changes in response to hypoxia[4]. The finding of general interaction patterns between HIFs and other transcription factors is restricted to specific experimental paradigms such as specific cell models[5].

5.2 Biological relevance of the work

5.2.1 Architecture and regulation of the *PHD2* (*EGLN1*) gene

The *PHD2* gene is located on chromosome 1 and consists of 5 exons[6]. In 2005 two different promoter sites upstream of the *PHD2* coding sequence were identified[7]. While the promoter element situated further upstream seemed to be dormant in adult tissue, the *PHD2* gene appears to be solely transcribed by the functionally active

downstream promoter. This latter promoter is reported to be embedded in a CpG island immediately 5´ of the translational start site and contains one functional HBS (-412 bp to the translational start site).

Although Metzen *et al.* initiated important work in light of the *PHD2* promoter analysis the transcriptional start site of the *PHD2* gene is still unknown. Then as now the analysis of ESTs was performed for the identification of the transcription start site, but did not succeed[7]. The ESTs listed in the UCSC genome browser were mostly located on the very 3'-end of the mRNA. The EST found most upstream is located at the 3'-end of the first exon and misses the mRNA sequence of almost the complete first translated exon. Thus, transcription start site prediction based on ESTs resulted mostly in functional questionable locations. The inbuilt disadvantage of this transcription start prediction method is the origin of ESTs itself. The single sequencing reactions might be erroneous and the first roughly 20 bases are often not reliable due to the polymerase / primer establishment that impairs the sequencing in the beginning of the reaction. However, these first bases are of importance when ESTs are used for transcription start prediction as they represent the very beginning of a transcribed sequence. As a consequence, these flaws lower the reliability of the results obtained. However, one potential transcription start site was found with the additional help of the detection of clusters of predicted transcription factor binding sites. This putative transcription start site lies in front of the first exon. This sequence was absent in the 3' truncated *PHD2* promoter construct P2P (-1070/-318). But the reporter was still transcriptionally active. This could mean that either the approach of deleting parts of the *PHD2* promoter in a reporter assay context is not appropriate or there is another, not yet predicted transcription start site present in the *PHD2* promoter. Eventually, a successful establishment of 5'-RACE is needed to confirm by experimental analysis the transcriptional start site.

This work provides evidence on an endogenous level that the *PHD2* promoter is hypoxically regulated primarily through HIF-1α rather than HIF-2α. This might be constrained to an intermediate time of acute hypoxia, since it is to date widely accepted that HIF-1α levels decrease with prolonged hypoxia while HIF-2α stabilization increases[8]. Thus, HIF-2α might take a more dominant role in PHD2 expression under specific conditions. Additionally, this work was performed in MCF7

cells that were reported to express a HIF-2α isoform with little transcriptional activity and influencing by this the recruitment to an endogenous transcriptionally active locus[9]. However, the finding is still in line with various other reports where by indirect means the prevalence of HIF-1 was proven to hypoxially induce PHD2 expression[10]. Further supportive data are given by the location of the HBS that is found close to the gene. This is a circumstance that statistically favors HIF-1 over HIF-2 binding[11,12].

Interestingly, we found a 55-95 bp encompassing stretch around the *PHD2* HBS conserved in humans, mice and other organisms. Despite the shortness of these regions they were able to confer hypoxic inducibility. When motifs located 5'- or 3'-adjacent to the HBS were disrupted by introducing a subset of mutations, hypoxic inducibility was completely lost, even though the functional HBS, previously shown to be sufficient for HIF-1 binding, was preserved[13]. The conserved *PHD2* promoter region also encompasses several consensus binding sequences for the ubiquitous transcription factor specificity protein 1 (Sp1) that assures transcription of many genes involved in early development of organisms. In Sp1-deficient cells PHD2 expression was not affected either on basal or on hypoxic levels, concluding that an accumulation of SP1-binding sites (5'-GGGCGG-3') only was due to the fact that the *PHD2* promoter is embedded in a GC-rich region. If not for SP1 other (co-) transcription factors might have functional relevance to *PHD2* transcriptional regulation. For instance, we showed that the ETS-transcription factor ETS-transcript variant (ETV) 4 and its structurally close subfamily member ETV5 are able to enhance the *PHD2* promoter activity in a HBS-dependent manner upon overexpression. Nonetheless, the increase of promoter activity is most probably not due to direct binding of those ETS-factors to the DNA-elements but rather owned to complex formation with HIF[14].

5.2.2 Applicability of the synthetic transactivation screening approach

The identification of HIF1/2α interaction partners was diverse in the past. Especially, yeast-two-hybrid (Y2H) screens were favored for long due to their low dependency on the physiological abundance or half-life of the interacting candidate protein. A Y2H screening in our research group lead to the identification of FKBP38[15], Cdr2[16] and

HELZ[17] as specific PHD interactors determining PHD2 protein stability, attenuating the hypoxic response in the cell or influencing general translational or proliferative activities. However, the discovered interactor HELZ notably was not linked to any cellular processes in hypoxia[17]. Another disadvantage of Y2H screenings is the circumstance that some interactions are based on post-translational modifications of the potential candidate protein. For example, the HIF-α-VHL interaction needs the hydroxylation of specific prolyl-residues in the HIF-α subunit. However, these post-translational modifications cannot be introduced by yeast and consequently prevent the detection of interacting proteins. Additionally, proteins formed from subunits might not be represented in their native confirmation causing misleading interaction of false positive proteins.

In comparison, proteomics strategies relying on affinity purification formats, such as immunoprecipitation followed by mass spectroscopy, to identify candidate proteins are freed from those drawbacks[18]. However, they come with other inbuilt disadvantages. First, the possible low abundance of target proteins is limiting the list of detectable interacting proteins. Second, the short half-life of candidate proteins in mammalian cells, such as HIF-α, is hindering the discovery of new interaction partners by proteomic approaches.

Another high-throughput screening is the use of genome-wide RNAi-mediated array experiments. They allow a loss-of-function screening, but miss to find the potential interaction of oncogenes that are usually overexpressed. Besides, the cost to perform such an assay are very high.

This work strived to identify interaction partners of HIFs that act in a broad trans-activating range. Therefore, we designed a cost-efficient, novel screening approach through the combination of overexpressing arrayed transcription factors together with the core *PHD2* promoter. We aimed to overcome the above mentioned disadvantages of a Y2H approach and the limitation of proteomics by allowing expression and post-translational modification in mammalian cells. Further, the screening was designed in such a way that we detect for a gain-of-function and not for the loss-of-function. With this approach we were able to identify HBS-specific transcription factor interplays. In this screening approach comprising 704 different transcription factors we found several members of the activating protein-1 (AP-1) family such as the proto-oncogenes JUN and FOSB as novel activators of the *PHD2* gene, which beforehand have only been reported to generally enhance hypoxic gene

expression[5]. Additionally, three ETS-family members were shown to increase *PHD2* promoter activity. Amongst them, ETV4 showed most striking effects on HBS-dependent transcription not only on the *PHD2*, but also on the *transferrin* promoter. This work demonstrates for the first time that ETV4 is interlinked with HIF-dependent transcription.

The previously described synthetic transactivation screening proved to be a powerful tool for HIF-dependent gene expression studies. It could be demonstrated that novel interactions amongst common signaling pathways were discovered and could be suggested for further use in areas like small molecule library analyses and genome-wide gene silencing approaches. Especially, the possible involved post-translational modification and the very short half-life of ETV4, seen in proteasome-inhibited or DMOG-treated cells, would have made the detection by Y2H or proteomics difficult. Most probably these are the reasons why ETV4 was not earlier found to be an interaction partner of HIF. By *in silico* comparison of the human p300/CBP interactome with the synthetic transcription factor overexpression array we found no simple redundancy of p300/CBP interactors with reproducible activators of the *PHD2* promoter. This could be due to a need for distinct target gene architectures that have been reported central for p300/CBP complex formation[19]. The lack of mere redundancy between the p300/CBP interactome and our screening results is of particular importance with regards to our screening design, as it provides some reliability regarding the specificity and its general applicability. Since ETV4 mRNA levels remain unchanged in hypoxic treatment, it could not be detected by genome-wide expression analyses which relies on comparative profiling of target mRNA expression levels. As a consequence, the link to a hypoxically regulated ETV4 family remained undiscovered until our development of the synthetic transactivation screening. The setup of this method facilitates easy adaptation in order to study other transcriptional pathways while combining a multiplexed single-well readout system with minimized inter-well variances that are usually difficult to control.

5.2.3 ETV4's hypoxia-induced target genes

It could be shown in gene array analyses that ETV4 facilitates HIF transactivation activity of 7.7% (47 out of 608 genes) of all hypoxically induced genes in PC3 human

prostate cancer cells - a cell line that shows high endogenous ETV4. Moreover, out of 61 established HIF target genes previously published the hypoxic induction of 14 genes was at least two-fold higher in the presence of ETV4 than in its absence[20]. These findings suggest a broad, but specific role of ETV4 which was so far unrecognized in the literature.

Interestingly, carbonic anhydrase 9 (*CA9*) was found to be strongly ETV4-dependent. The strong hypoxic inducibility of *CA9* might be interlinked with the additional presence of ETV4 in cooperation with HIF and ATF-4[21,22]. Intriguingly, the architecture of the *CA9* HBS with the 5' and 3' adjacent region is similar to the ones of the *PHD2* or *transferrin* promoter. We could also demonstrate a super-induction of the *CA9* promoter by ETV4 overexpression, whereas this effect was abrogated when using HBSs with dissimilar flanking regions to the *PHD2* HBS. We therefore hypothesize that HBS-architecture is a strong driver of ETV4-HIF synergism. However, direct interactions of ETV4 with HBS neighboring DNA sequences seem to be unlikely out of two reasons. First, the commonly known consensus sequence of the ETV4 subfamily is AT-rich and is not found in the GC-rich vicinity of the HBS. Secondly, although the ETV4 family members are highly similar in DNA-binding domain structure ETV1 was demonstrated not to potentiate the *PHD2* or *transferrin* promoter activity. Suggesting that rather sterical reasons - possibly generated by a specific DNA-sequence - determine ETV4-dependent hypoxia-induced genes from ETV4-independent ones.

Our findings showed activation of the *PHD2* promoter in the U2OS cell model when ETV4 was overexpressed. However, an ETV4 knockdown did not influence PHD2 expression levels in U2OS cells. It seems that the osteosarcoma cell line U2OS is not an adequate cell model for investigating the effects of PHD2 expression, since ETV4 mRNA and protein expression levels are very low in those cells. In addition, it is well possible that the overlapping features of the ETV4-subfamily compensate for the transient or stable knockdown of ETV4 and thus, do not affect *PHD2* promoter activity or expression levels in U2OS cells. To verify a putative compensation we tested ETV1 and ETV5 mRNA levels in the U2OS knockdown cells. ETV5 is more expressed than ETV1, but if ETV5 protein is sufficiently expressed for compensating ETV4 remains unknown. An ETV4 knockdown in a cell line such as PC3 that expresses substantially higher levels of ETV4 surprisingly also did not show the

expected decrease in hypoxic PHD2 expression, but rather in PHD3 expression levels. Individual inspection of the genome-wide expression analysis revealed a 2.7-fold hypoxic induction of PHD2 that was only reduced by 16% in the absence of ETV4. This indicates that PHD3 rather than PHD2 is the main molecular oxygen sensor in this prostate cancer cell type. To further understand the function of ETV4 in hypoxic tissue it would be interesting to study, if PHD3 levels correlate with ETV4 expression in prostate cancer tissue sections.

5.2.4 The trimeric HIF:p300/CBP:ETV4 complex

Intrigued by the HIF-dependency of ETV4 effects, we characterized the biochemical interaction between ETV4 and HIF-α. In a mammalian two-hybrid approach we found that ETV4 co-operates with the C-terminal activation domain of HIF-1α to transactivate target genes. This interaction is still sensitive to oxygen although the oxygen-dependent degradation domain of HIF-1α is missing. A very close interaction between ETV4 and HIF-1/2α of approximately 5.6-5.7 nm by FRET experiments suggests per se direct interaction between ETV4 and HIFs. Despite this very close proximity, it is still possible that another protein might be involved.

We rather suggest a ternary complex formation between ETV4 and HIF-α via p300/CBP. Based on the following results: first, we could show that the interaction between ETV4 and HIF-1α could be competed by the co-transfection of CBP/p300-interacting transactivator 2 (CITED2), known to negatively regulate HIF function[23,24]. Structural analyses revealed that CITED2 and HIF-1α share an overlapping binding interface on the 300-kilodalton co-activator protein (p300) cysteine-histidine-rich 1 (CH1) domain and competition assays showed a 33-fold higher affinity of CITED2 for binding to p300 CH1 than a corresponding HIF-1α CAD peptide, indicating that CITED2 is a dominant inhibitor of HIF-1α:p300/CBP complex formation[25]. We could further underscore our model by the knockdown of p300 or FIH. Through a transient knockdown of p300 though, we found only a slight reduction of the intrinsic transactivation activity that was mediated by the C-terminal activation domain accompanied by a robust superinduction through ETV4. We therefore hypothesized that ETV4 activation occurs independent of the presence of p300. Mechanistically, this could be explained by compensatory action of CREB binding protein CBP protein

that substitutes for the loss of p300. FIH is an oxygen-dependent negative regulator of HIF transcriptional activity as it hydroxylates a distinct asparagine residue within the HIF-α carboxy terminus in normoxic conditions. The hydroxylated HIF-α carboxy terminus is no longer able to associate with p300/CBP. A transient depletion of factor inhibiting HIF (FIH) resulted in the steady association of HIF with p300/CBP. The FIH knockdown interrupted the oxygen sensitivity of the ETV4-HIF-1α cooperation and adds to the hypothesis that the HIF-ETV4 interaction is influenced by another protein, namely p300/CBP[26].

In conclusion, although FRET data propose a direct interaction of HIF and ETV4, our findings suggest a ternary complex formation with HIF-1α binding to both p300 domains: CH1 and CH3[27]. ETV4 has been reported to interact with p300 at its CH3 domain and could come by this into close contact with HIF-α[28].

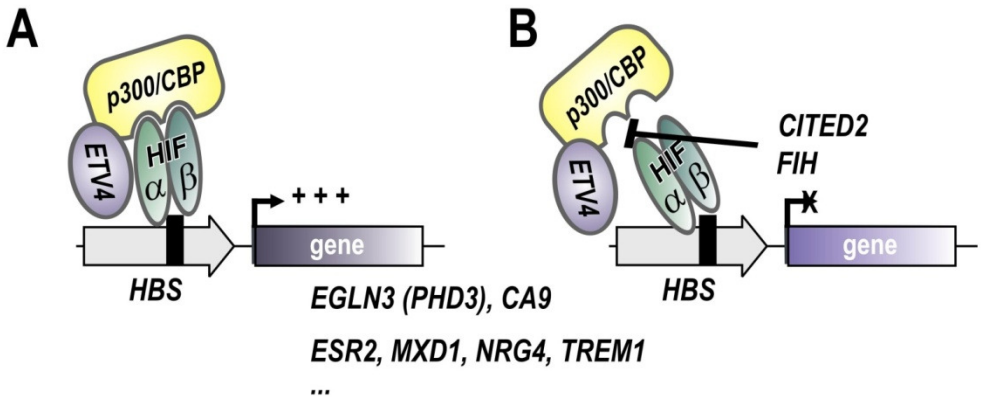

Figure 1. Transcriptional cooperation between ETV4 and HIF-1 is disrupted by CITED2 or FIH. **(A)** Scheme of the potential interactions between HIF-1, p300/CBP and ETV4 as assessed by mammalian two-hybrid assays. **(B)** Both CBP/p300-interacting transactivator 2 (CITED2), known to negatively regulate HIF function and factor inhibiting HIF (FIH) can block the interaction between HIF-1α and p300/CBP and as a result limit the synergistically driven transcription of HIF:ETV4 downstream target genes (*EGLN3*, *CA9*, etc.).

5.2.5 HIF and ETV4 – a toxic duet in cancer

The HIF and ETV4 pathways interleave partially with each other. For example, various matrix metalloproteases (MMP) like MMP-1, MMP-3, MMP-7 and MMP-9, as well as the chemokine receptor CXCR4, the NO-synthetase iNOS and the

cyclooxygenase COX-2 are common target genes[29–39]. As a consequence, the malignant properties of several cancer cells could be due to the detrimental cooperation of HIF and ETV4 on regulatory regions of the aforementioned genes.
Importantly, not only ETV4 but also its structurally-close relatives ETV1 and ETV5 are reported to be frequently overexpressed in prostate cancer due to their translocation and fusion to androgen-responsive genes[40–42]. For example, the loss of the important tumor suppressor phosphatase and tensin homolge (PTEN) was observed to cause an accumulation of HIF-1α also in normoxia – a phenomenon often observed in invasive prostate cancers[43]. In recent animal studies, the early loss of PTEN combined with a simultaneous overactivation of ETS-factors lead to a gross activation of target genes necessary for prostatic cancer development[44,45]. From a therapeutic view point, it would be highly informative to elucidate the role of HIF/ETV4 interaction in these pathologies. We therefore suggest to extend tissue microarray analyses from breast cancer samples[14], to other clinical specimens such as prostate cancer tissue. This would not only shed light on the HIF/ETV4 interaction in additional cancer types, but also allow a systematic analysis of the coincidence of elevated HIF and ETV4 levels in the nuclei of these cells.

5.2.6 Developmental aspects of the HIF and ETV4 collaboration

In developmental aspects, ETV4 has been involved in branching morphogenesis of kidney and liver as well as in spinal motor neuronal differentiation[46–50]. Notably, the ETS-domain responsible for DNA-interaction shares 95% of similarity and the transactivation domains still roughly 85% amongst all ETV4-subamily members[51–55]. The structural similarities of ETV4 family members ETV1 (ER81) and ETV5 (ERM) raised the question if these factors have overlapping functions in tissue development and cancer progression[55]. ETV4 and ETV5 show indeed strongly compensatory functions in kidney development that were only revealed when both transcription factors had been knocked out[46,47].

This familiarity led us to test the additional sub-family members of ETV4. Notably, we could show that not only ETV4, but also ETV5 super-induced the hypoxic *PHD2* and *transferrin* promoters in an HBS-dependent manner, whereas ETV1 did not. This

suggests a putative functional overlap of the two subfamily members in the hypoxic gene regulation. As not all ETV4-subfamily members show superinducibility with HIF, it is probable that the interface of HIF-α interaction rather lies outside of the ETS-DNA-binding domain. As tissue hypoxia often occurs in developmental processes, our findings suggest a physiological role of ETV4/ETV5 in low oxygenated tissues or organs at the beginning of vascularisation. In line with the observations that ETV4 is highly expressed in various forms of cancer and that hypoxic tumors show high HIF-1 expression levels, we found an impressively good correlation between ETV4 and PHD2 protein levels in breast cancer tissues[51,56]. This hints to a potentially relevant function of ETV4 in hypoxic tissues *in vivo*. In contrast to ETV1 and ETV5, ETV4 is more widely expressed in most tissues (such as the kidneys, mamma and prostate) and makes it an interesting target for further studies on EPO-producing cells or hypoxic breast or prostate cancer. Even though a group effect of the entire subfamily on hypoxic gene regulation would be supportive, the lack of high affinity and specificity antibodies momentarily inhibits further investigations.

5.2.7 ETV4 is oxygen regulated and interacts indirectly with PHD2

In line with the ETV4-PHD2 correlation in arrayed tissue samples, our data also suggest a positive correlation between ETV4 and PHD2 mRNA expression levels in various cancer cell lines, such as U2OS and HepG2. However, mRNA levels may differ from protein data - as shown in the prostate cancer cell line PC3. This cell line was reported to express only intermediate levels of ETV4 mRNA, but ETV protein levels are elevated due to an impaired degradation process via ubiquitin ligase COP1[44].

We found in U2OS osteosarcoma cells that ETV4 protein levels are up-regulated in hypoxia, but mRNA levels remain unchanged suggesting translational stability changes on the protein level. The ETV4 and HIF pathways show an interesting pattern of overlap: as HIF-1α, ETV4 shows an accumulation of protein by proteasomal inhibition with MG132, after the treatment with the PHD-inhibitor dimethyloxalylglycine (DMOG) or in hypoxia[57]. However, differences are: ETV4 levels are high at the beginning of hypoxia-mimicking DMOG treatment, whereas HIF-1α is

induced slightly later. Eventually both factors degrade in prolonged hypoxia-mimicking conditions. Since PHD2 and PHD3 are part of the negative HIF-feedback loop, their increased expression in hypoxia partially compensates the decreased enzymatic activity during oxygen-deprivation and consequently ensures basal proteasomal degradation of HIF-1α[58]

It is widely accepted that PHDs regulate the stability of HIF-α subunits through the hydroxylation of distinct proline residues[59–63]. The PEA3 subfamily, similar to HIF-α, has also been reported to undergo diverse posttranslational modifications such as phosphorylation or SUMOylation and might also include hydroxylation[64–79]. ETV4 migrates as multiple distsinct bands in SDS-PAGE could reflect posttranslational modifications of the same protein. Additionally, PHDs were reported to putatively hydroxylate other proteins than HIF-α, such as RbpI, the large subunit of RNA polymerase II, activating transcription factor-4 (ATF-4) or pyruvate kinase M2 (PKM2)[22,80–82]. This list could be enlarged to comprise ETV4 and might explain the hypoxic accumulation of ETV4. We could demonstrate that ETV4 indirectly interacts with PHD2 in hypoxia and potentially in normoxia in a mammalian-2-hybrid approach. In line with this speculation, the application of a PHD-inhibitor (DMOG) or the knockdown of the main cellular oxygen sensor PHD2 lead to increased ETV4 protein levels in oxic U2OS cells.

In PC3 cells ETV4 downregulation decreased *PHD3* mRNA induction in hypoxia, suggesting that ETV4 also positively regulates PHD3 expression. It could be hypothesized that COP1 - similar to the von-Hippel-Lindau protein - is not able to degrade ETVs in hypoxic conditions due to the missing hydroxylation of distinct prolines. This would suggest the implication of PHDs in the regulation of ETV4 degradation. The study of a group effect, namely the knockdown of PHD1 and PHD3 in U2OS cells could reveal if other PHDs are equally involved in ETV4 regulation. The enzymatic PHD effect on the surrogate substrate protein ETV4 is hindered by the proline-rich nature of the protein that would probably mask the detection of hydroxylated prolines by mass spectroscopy[83]. Finally, we cannot exclude that the increased ETV4 protein levels could also be due to translation-enhancing mechanisms that influence the ETV4 turnover.

5.3 Outlook

Since ETV4 and its family members just entered the large field of hypoxic transcriptional regulation a detailed mechanistic insight of their interaction with HIF and possibly PHDs premains elusive. More work is required to investigate how the ETV4-subfamily members play a role in influencing the hypoxic pathway. Most urgently, the origin of the potential hypoxic regulation of ETV4 and its concise mechanism needs to be elucidated. More insight could be gained through the use of other hypoxia-mimicking agents such as cobalt chloride or desferrioxamine that need to be applied to pin down the origin and regulatory mechanisms of an ETV4-HIF synergism. Importantly, we aim to find out what common features are needed for driving ETV4 to be a co-factor of HIFs. This could be done in models for breast or prostate cancer, without neglecting developmental questions where the ETV4-subfamily is known to be crucially involved. As a matter of fact, the implications of development in the kidney and liver are most intriguing as they represent important set points of physiological hypoxia sensing and response. An exciting approach would be the exposure of ETV4-null mice to normobaric hypoxic conditions accompanied by a tight monitoring of transcriptional and metabolic changes (i.e. EPO-production) in organs and other tissues, e.g. kidney, liver, lung as well as breast tissue and the prostate gland since ETVs and HIFs showed here to play a crucial role in tumor development.

Furthermore, the analysis of the synthetic transactivation screening could be enlarged from activators to inhibitors. So far, only activators were analyzed whereas potential repressors of PHD2 expression had been neglected in this screening approach. This could open a completely new level of regulatory transcription mechanisms in the hypoxic cascade, since HIF is only known to activate but not to directly repress gene transcription.

Finally, the adaptation of the established screening approach could be applied to small molecules or siRNA-mediated library screenings where downstream targets of HIF are analyzed in detail.

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6 Contributions to publications and manuscripts

1. **Wollenick K**, Hu J, Kristiansen G, Schraml P, Rehrauer H, Berchner-Pfannschmidt U, Fandrey J, Wenger RH and Stiehl DP
"Synthetic transactivation screening reveals ETV4 as broad co-activator of hypoxia-inducible factor signaling" *Nucleic Acid Research (2011)*
All figures except figure 5 and subfigure 7 D-E.

2. Stiehl DP, Bordoli MR, Abreu-Rodríguez I, **Wollenick K**, Schraml P, Gradin K, Poellinger L, Kristiansen G, Wenger RH
"Non-canonical HIF-2α function drives autonomous breast cancer cell growth via an AREG-EGFR/ErbB4 autocrine loop" *Oncogene (2011)*
Cloning, purification and pre-testing of the human *PHD2* promoter pGL(P2P) reporter and the hypoxia-responsive reporter pSVL(P2P) (95bp) in subfigure 4 C-D.

3. Lehmann S, Stiehl DP, Honer M, Dominietto M, Keist R, Kotevic I, **Wollenick K**, Ametamey S, Wenger RH and Rudin M
"Longitudinal and multimodal in vivo imaging of tumor hypoxia and its downstream molecular events" *Proc Natl Acad Sci U S A (2009)*
Cloning and pre-testing of the firefly luciferase reporter vector pGL(P2P)95bp (HRE-luciferase) used in figure 2, 3 and 5.

7 Curriculum vitae

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Publications

4. **Wollenick K**, Hu J, Kristiansen G, Schraml P, Rehrauer H, Berchner-Pfannschmidt U, Fandrey J, Wenger RH and Stiehl DP; "Synthetic transactivation screening reveals ETV4 as broad co-activator of hypoxia-inducible factor signaling" *Nucleic Acid Research (2011)*
3. Stiehl DP, Bordoli MR, Abreu-Rodríguez I, **Wollenick K**, Schraml P, Gradin K, Poellinger L, Kristiansen G, Wenger RH; "Non-canonical HIF-2α function drives autonomous breast cancer cell growth via an AREG-EGFR/ErbB4 autocrine loop" *Oncogene (2011)*
2. Lehmann S, Stiehl DP, Honer M, Dominietto M, Keist R, Kotevic I, **Wollenick K**, Ametamey S, Wenger RH and Rudin M; "Longitudinal and multimodal in vivo imaging of tumor hypoxia and its downstream molecular events" *Proc Natl Acad Sci U S A (2009)*
1. Duke-Cohan JS, **Wollenick K**, Witten EA, Seaman MS, Baden LR, Dolin R, Reinherz EL;"The heterogeneity of human antibody responses to vaccinia virus revealed through use of focused protein arrays." *Vaccine (2009)*

Talks

1. "Feedback regulation of PHD oxygen sensor", **Respiratory Physiology Meeting**, Zurich, Switzerland, January **2009**

Conferences & poster presentations

4. "A transcription factor screening identified ETV4 as novel co-activator of HIF-1",
   7th Symposium of the Zurich Center for Integrative Human Physiology (ZIHP), Zurich, August **2009**
3. "HIF dependent and independent transcriptional regulation of the human *PHD2* promoter"
   HypoxiaNet COST meeting, Davos, Switzerland, January **2011**
2. "HIF dependent and independent transcriptional regulation of the human *PHD2* promoter",
   Hypoxia: Molecular Mechanims of Oxygen Sensing and Response Pathways, Keystone Resort, Colorado, USA, January **2010**
1. "Basal and inducible regulation of the human *PHD2* promoter",
   EurOxy: Tumour Hypoxia: From Biology to Therapy II, Monsummano Terme, Tuscany, Italy, October **2008**

Personal skills & competences

Languages German (native), English (fluent), French (proficient), Spanish (basic)

Teaching Assistance in practical courses in physiology for medical and human biology students (2007-2010)

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